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PRINCIPAL INVESTIGATOR: John G. Koland, Ph.D.

CONTRACTING ORGANIZATION: University of Iowa
Iowa City, Iowa 52242

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Iowa City, Iowa 52242

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The role of the ErbB3 protein in breast cancer cell proliferation was examined. In the first year of funding, it was demonstrated that the ErbB3 protein, although possessing a protein tyrosine kinase (PTK) homology domain, is in fact devoid of intrinsic PTK activity. In the second year of funding, we demonstrated the critical dependence of heregulin signaling upon the PTK activity of the ErbB2 protein, and characterized the interaction of ErbB3 with phosphoinositide (PI) 3-kinase in breast cancer cells. In the third year of funding, we identified the tyrosine residue in ErbB3 responsible for binding the Shc adapter protein, clarified the role of Shc in activation of the Ras/mitogen-activated protein kinase (MAPK) pathway, and began an exhaustive examination of other tyrosine residues in the ErbB3 C-terminus. In the fourth year of funding, we characterized the role of six distinct tyrosine residues within consensus PI 3-kinase binding sites in mediating interactions with PI 3-kinase and activating the downstream target Akt, a protein kinase known to be involved in intracellular signaling, but not previously implicated in the signaling pathways of the ErbB3 receptor.

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FOREWORD

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John D Holland 8/29/98

PI - Signature Date

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INTRODUCTION

Breast cancer cells have been observed to express abnormally high levels of receptor proteins in the ErbB family, which includes the EGF receptor, ErbB2, ErbB3 and ErbB4 (also designated as HER1-HER4, respectively) (4, 11, 12, 14, 16). High levels of EGF receptor and ErbB2 expression in tumor cells have been considered indicators of poor prognosis (9). Given that these receptors activate mitogenic signaling pathways, it is possible that they play a role in the abnormal proliferation of breast cancer cells. The polypeptide heregulin (or Neu differentiation factor) (1) is secreted from breast cancer cells (8), and has been shown to activate ErbB2, ErbB3 and ErbB4 receptor proteins (2, 3, 13, 18). Indeed, ErbB2 and ErbB3 have been shown to function together as a coreceptor for heregulin (17). Studies completed in the first year of funding had demonstrated that the ErbB3 protein, unlike other ErbB family members, is actually devoid of intrinsic protein tyrosine kinase (PTK) activity (15). This and subsequent work was facilitated by our cloning of the rat ErbB3 cDNA (7). In the second year of funding, these studies were extended to demonstrate that in ErbB2/ErbB3 coreceptors, the ErbB2 protein provides the PTK activity necessary for signal transduction (10). Also, the yeast two-hybrid system was used to investigate the mechanism of interaction between the ErbB3 protein and the signal-transducing enzyme phosphoinositide (PI) 3-kinase (6). Efforts in the third year of funding focused on examining of the mechanism of activation of the Ras/mitogen-activated protein kinase (MAPK) pathway by ErbB2/ErbB3 heregulin coreceptors (19). In the fourth year of funding, we have at the suggestion of previous reviewers of our progress reports continued to investigate the signal transduction mechanisms of ErbB2/ErbB3 heregulin coreceptors. In particular, we have examined the interaction between heregulin coreceptors and the PI 3-kinase signaling enzyme and the consequence of this interaction in mitogenic signaling.

BODY

I. Manuscripts published in the fourth year of funding

In the fourth year of funding, three studies initiated in the first three years of funded work were concluded, which resulted in our successful publication of the three manuscripts listed below (see Appendices).

1. Hellyer, N.J., Cheng, K., and Koland, J.G. (1998) "ErbB3 (HER3) Interaction with the p85 Regulatory Subunit of Phosphoinositide 3-Kinase," *Biochem. J.* **333**, 757-763.
2. Kim, H.-H., Vijapurkar, U., Hellyer, N.J., Bravo, D., and Koland, J.G. (1998) "Signal Transduction by Epidermal Growth Factor and Heregulin via the Kinase-deficient ErbB3 Protein," *Biochem. J.* **334**, 189-195.
3. Vijapurkar, U., Cheng, K., and Koland, J.G. (1998) "Mutation of a Shc Binding Site Tyrosine Residue in ErbB3/HER3 Blocks Heregulin-dependent Activation of Mitogen-activated Protein Kinase," *J. Biol. Chem.* **273**, 20996-21002.

The work leading to the first manuscript was described in detail in the Annual Report for the second year of funding (submitted September 1995), except for an additional experiment performed in the fourth year of funding. In this experiment, we tested the hypothesis that a proline-rich candidate SH3 domain binding sequence found within the C-terminus of the ErbB3 protein participated in interactions with the p85 regulatory subunit of PI 3-kinase. This was initially suggested by our yeast two-hybrid studies as described in the second Annual Report. Here we demonstrated by an *in vitro* binding assay (see Fig. 1) that the ErbB3 protein could interact with the isolated SH3 domain of p85. Hence, the ErbB3 protein possessed multiple sequence elements [six Tyr-Xaa-Xaa-Met (YXXM) motifs and a unique proline-rich sequence] capable of interacting with the SH2 and SH3 domains of the p85 regulatory subunit of PI 3-kinase. The results of our yeast two-hybrid system studies and this *in vitro* binding experiment, which comprised the first manuscript listed above, presented a novel mechanism by which the ErbB3 protein might interact with and regulate the activity of PI 3-kinase. Subsequent experiments tested this mechanism through studies of recombinant receptor proteins expressed in cultured cells (see below).

The work leading to the second publication was previously described in the Annual Report for the first year of funding (submitted September 1995). For technical reasons we were forced to revise our manuscript and provide additional control experiments that significantly delayed publication of our findings. However, the resulting paper is the first definitive characterization of the role of protein tyrosine kinase activity in signal transduction by ErbB family coreceptor complexes. In particular, it examines the consequences of the lack of intrinsic protein tyrosine

kinase activity in the ErbB3 protein upon signal transduction mediated by EGF receptor/ErbB3 and ErbB2/ErbB3 coreceptor complexes. This study nicely complemented our previously published *in vitro* biochemical characterization of the ErbB3 protein [(15), see Appendices], in which ErbB3 was shown to be devoid of intrinsic protein tyrosine kinase activity [see also (5)].

Work leading to the third published manuscript was described in detail in the Annual Report for the third year of funding (submitted September 1997). Here we documented our demonstrations that (1) a specific tyrosine residue in the C-terminus of ErbB3 was responsible for its interaction with the Shc protein, (2) this interaction with Shc resulted in the activation of the mitogen-activated protein kinase (MAPK) signal transduction pathway, and (3) the Shc/MAPK signaling pathway contributed to the stimulation of DNA synthesis occurring upon activation of ErbB2/ErbB3 heregulin coreceptors. Because the blockade of the Shc/MAPK signaling pathway did not entirely block the stimulation of DNA synthesis by the heregulin coreceptor, we suspected that the PI 3-kinase pathway was also involved in its mitogenic signaling. Hence, in the fourth year of funding we have continued to examine the mechanism and consequences of PI 3-kinase interactions with the ErbB3 protein (see below).

II. Interaction of the ErbB2/ErbB3 heregulin coreceptor with PI 3-kinase and its downstream effectors

Because the ErbB3 protein is unique among EGF receptor/ErbB family members in that its C-terminal phosphorylation domain contains a large number (12) of candidate tyrosine residue phosphorylation sites, we have endeavored to explore the roles of these various tyrosine residues by tyrosine to phenylalanine (Tyr→Phe) substitutions generated by site-directed mutagenesis. Efforts in the fourth year of funding have been directed towards the six C-terminal tyrosine residues within YXXM motifs that are candidate PI 3-kinase binding sites. Because the p85 regulatory subunit of PI 3-kinase contains two SH2 (Src homology 2) domains, each independently capable of binding to phosphorylated YXXM motifs, a variety of scenarios for the interaction between ErbB3 and p85 might be envisioned, some involving multiple ErbB3 YXXM motifs. Hence, we considered it would be necessary to generate a large number of Tyr→Phe mutants in which various combinations of the six YXXM motifs were altered. In our preliminary investigations, a cDNA encoding a mutant ErbB3 protein (ErbB3-6F) in which all of the six tyrosine residues in YXXM motifs were substituted with phenylalanine was constructed. Also, recognizing that interactions between ErbB3 and p85 could be potentiated by interactions between the ErbB3 proline-rich sequence and the SH3 domain of p85 (6), we created a cDNA encoding a mutant ErbB3 protein in which this proline-rich sequence element was deleted (ErbB3-Δpro). Wild-type ErbB3, ErbB3-6F and ErbB3-Δpro were co-expressed with the ErbB2 protein in COS7 cells to examine the signal-transducing abilities of ErbB2/ErbB3 heregulin coreceptors containing

the respective ErbB3 proteins (see Fig. 2A). We determined that each of the ErbB3 proteins was expressed in the COS7 cells, and each was phosphorylated on tyrosine residues in response to heregulin (see Fig. 2B). As predicted, the ErbB3-6F protein failed to interact with either the p85 subunit of PI 3-kinase (see Fig. 2B) or PI 3-kinase catalytic activity (see Fig. 2C). Interestingly, there was no apparent deficit in the ability of the ErbB3-Δpro protein to interact with PI 3-kinase, suggesting that the proline-rich sequence within the ErbB3 C-terminus was not necessary for high affinity ErbB3/p85 interactions, although the possibility that this proline-rich sequence element is involved in the regulation of PI 3-kinase activity was not ruled out. A major result of this investigation was the generation of a mutant ErbB3 protein (ErbB3-6F) that did not in the context of a heregulin-stimulated ErbB2/ErbB3 coreceptor interact with the PI 3-kinase enzyme. Hence we anticipate that this mutant receptor protein will in the future be of great value in investigating the role of PI 3-kinase in heregulin/ErbB receptor signal transduction.

In a related effort, we investigated the possibility that the ErbB2 protein in ErbB2/ErbB3 heregulin coreceptors might itself interact with and activate PI 3-kinase. We did observe that ErbB2 immunoprecipitates from heregulin-stimulated cells expressing ErbB2/ErbB3 coreceptors contained small amounts of the p85 protein as well as PI 3-kinase activity, although both were less that 10% of that found in ErbB3 immunoprecipitates (see Fig. 3). To explore this further, we examined the ability of an ErbB2 protein with a YXXM motif Tyr→Phe amino acid substitution (ErbB2-952F) to immunoprecipitate PI 3-kinase activity (see Fig. 4). To our surprise, the immunoprecipitation of PI 3-kinase with ErbB2 was not blocked by the amino acid substitution, but was blocked by the mutation of YXXM motifs in the ErbB3 protein. A straightforward interpretation of this finding is that the presence of PI 3-kinase activity in ErbB2 immunoprecipitates was due to residual coimmunoprecipitation of the ErbB3 coreceptor partner. Hence, in contrast to the conclusion of others, we find no evidence that ErbB2 physically interacts with PI 3-kinase. Thus, it appears that ErbB3 is unique among ErbB family members in its ability to interact with the PI 3-kinase signaling pathway.

Our final investigations addressed the question of whether the Akt protein kinase (also designated PKB), a known downstream effector of PI 3-kinase, is activated in response to stimulation of ErbB2/ErbB3 heregulin coreceptors. To this end we employed an *in vitro* kinase assay in which the Akt protein was immunoprecipitated from heregulin-stimulated cells expressing ErbB2/ErbB3 coreceptors, and its activation assayed with an Akt kinase-specific peptide substrate (see Fig. 5). Indeed the Akt kinase was activated in response to heregulin in cells expressing wild-type ErbB2/ErbB3 heregulin coreceptors. To investigate the role of the PI 3-kinase and Shc/MAPK pathways in the activation of Akt, we employed mutant ErbB3 receptor proteins unable to couple either with PI 3-kinase (ErbB3-6F) or the Shc protein (ErbB3-1325F). To our surprise, both of these mutant ErbB3 proteins were able to mediate heregulin-dependent Akt activation (see

Fig. 6). The possible mechanisms by which the Akt kinase might be activated by an ErbB3 receptor mutant unable to directly couple to PI 3-kinase include (1) activation by a Ras-mediated pathway activated by the ErbB2 coreceptor partner, and (2) activation via the Src protein tyrosine kinase, a known target of ErbB2 receptor activation. Because activation of the Akt protein kinase is known to promote cell survival via an inhibition of apoptotic signaling pathways, its role in the proliferation of breast cancer cells could indeed be significant, and investigation of Akt kinase activation by ErbB2/ErbB3 coreceptors will remain a subject of future investigation in this laboratory. A manuscript describing our investigations of the PI 3-kinase and Akt signaling pathways is in preparation.

CONCLUSIONS

Knowledge of the mechanisms of ErbB2/ErbB3 coreceptor signaling is crucial to our understanding of the control of breast cancer cell proliferation, as many of the cultured breast cancer cells examined to date express abnormally high levels of both of these receptor proteins. The heregulin polypeptide, which is an activating ligand for the ErbB2/ErbB3 coreceptor, is known to be expressed and secreted by breast cancer cells, and could therefore be critical to the proliferation of those breast cancer cells expressing the coreceptor. However, because of the complexity of heregulin/ErbB coreceptor interactions, our understanding of the signaling mechanisms of these receptor systems is yet limited.

Studies completed in the four years of research first investigated the role of protein tyrosine kinase activity in signaling by ErbB family coreceptors. Indeed it was determined that the ErbB3 protein was itself devoid of intrinsic kinase activity. Phosphorylation and activation of the ErbB3 protein in the cellular context was shown to result from the action of an associated ErbB family member. We have also begun to elucidate the role of the Shc/MAPK and PI 3-kinase pathways in signaling by the ErbB3 protein. It appears that under some conditions ErbB3 can play a dominant role in the recruitment of signaling molecules to ErbB coreceptor complexes. This contrasts the dominant role played by the ErbB2 protein tyrosine kinase in the phosphorylation of coreceptor constituents and associated signaling molecules. Additionally, we have generated a series of cDNAs encoding mutant ErbB3 receptor proteins that will be applied in future investigations of the mechanisms of mitogenic signaling and cellular transformation by ErbB2/ErbB3 heregulin coreceptors. The role of the Akt kinase, a newly identified effector of activated ErbB coreceptors, will certainly be investigated.

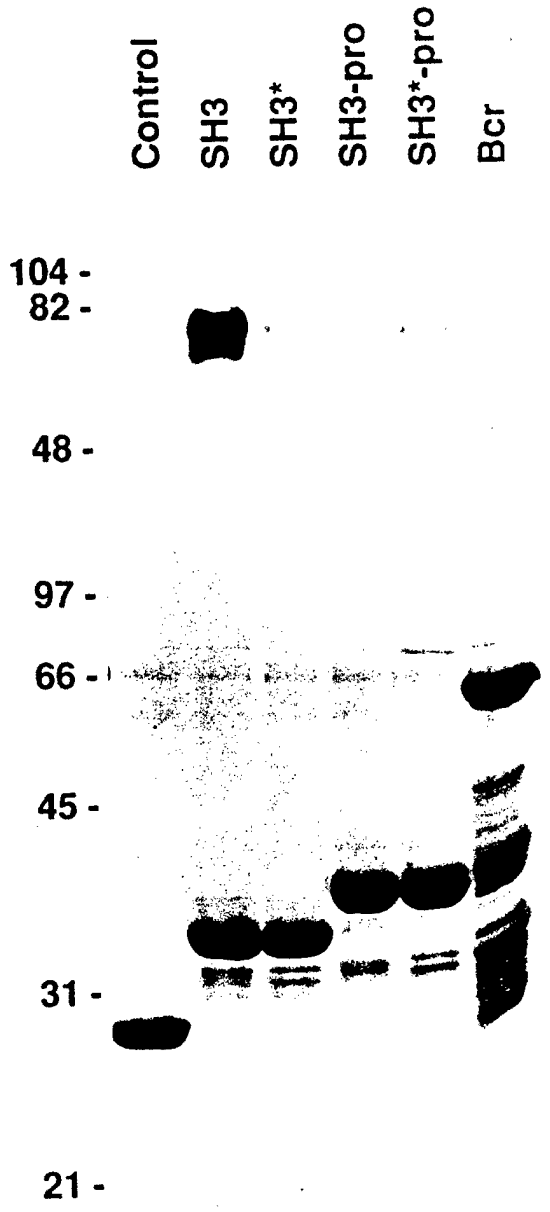

FIG. 1. ***In vitro*** **association of GST-p85-SH3 domain fusion proteins with ErbB3.** *A*, to investigate the role of the p85 SH3 domain in ErbB3/p85 interactions, glutathione S-transferase (GST) fusion proteins containing either the p85 SH3 domain (GST-SH3, amino acids 1-80), the mutant SH3-D21N domain (GST-SH3*), the SH3 domain with a small proline-rich region of the Bcr domain appended (GST-SH3-pro, amino acids 1-101), or the contiguous SH3 and Bcr domains (GST-SH3-bcr, amino acids 1-339) were generated. GST-p85 fusion proteins (2.0 nmol each) were incubated with a recombinant ErbB3 cytosolic domain protein (2.0 pmol), and samples of glutathione-agarose precipitates were subsequently resolved by SDS-PAGE. Association of the ErbB3 protein with precipitated GST or GST-p85 fusion proteins was detected by immunoblotting with an ErbB3-specific antibody. *B*, GST and GST-p85 fusion proteins (0.9 nmol each) analyzed by SDS-PAGE with Coomassie Blue staining.

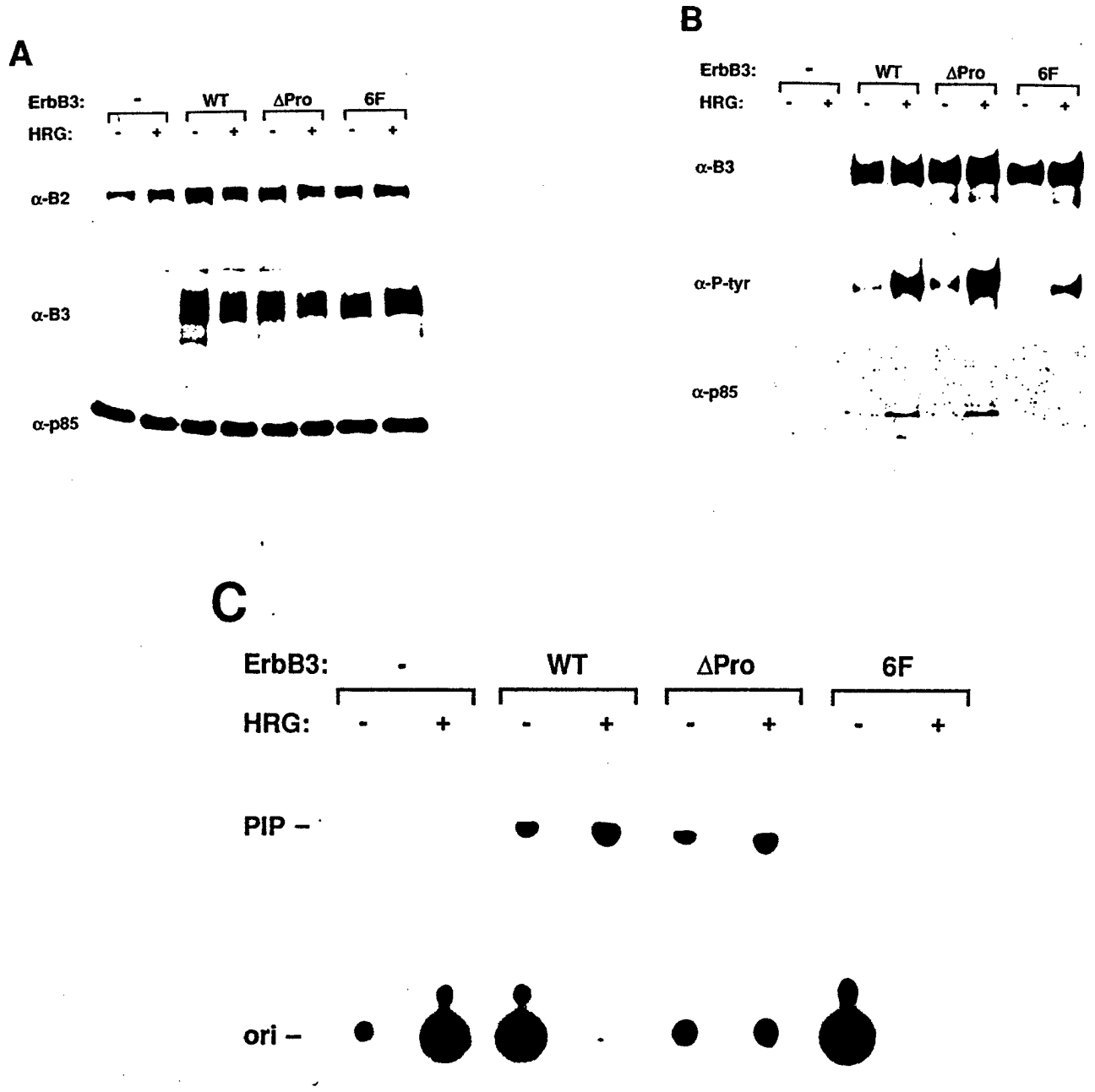

FIG. 2. **PI 3-kinase association with wild-type and mutant ErbB3 proteins.** COS7 cells were transiently cotransfected with the pcDNA3 vector incorporating the ErbB2 cDNA and either the parent expression vector (-) or vector incorporating wild-type ErbB3 (WT), ErbB3ΔPro (ΔPro), or ErbB3-6F (6F) cDNA as indicated. Transfected cells were treated for 20 min in the absence (-) or presence (+) of 1 nM heregulin and subjected to detergent lysis. *A*, cell lysates were immunoblotted with antibodies recognizing ErbB2 (α-B2), ErbB3 (α-B3) and p85 (α-p85) as indicated. *B*, lysates were immunoprecipitated with ErbB3-specific antibody. One-half of each immunoprecipitate was immunoblotted with antibodies recognizing ErbB3, phosphotyrosine (α-P-tyr), and p85. *C*, the remainder of each ErbB3 immunoprecipitate was analyzed for PI 3-kinase activity by a thin layer chromatographic assay. These results are representative of three independent experiments.

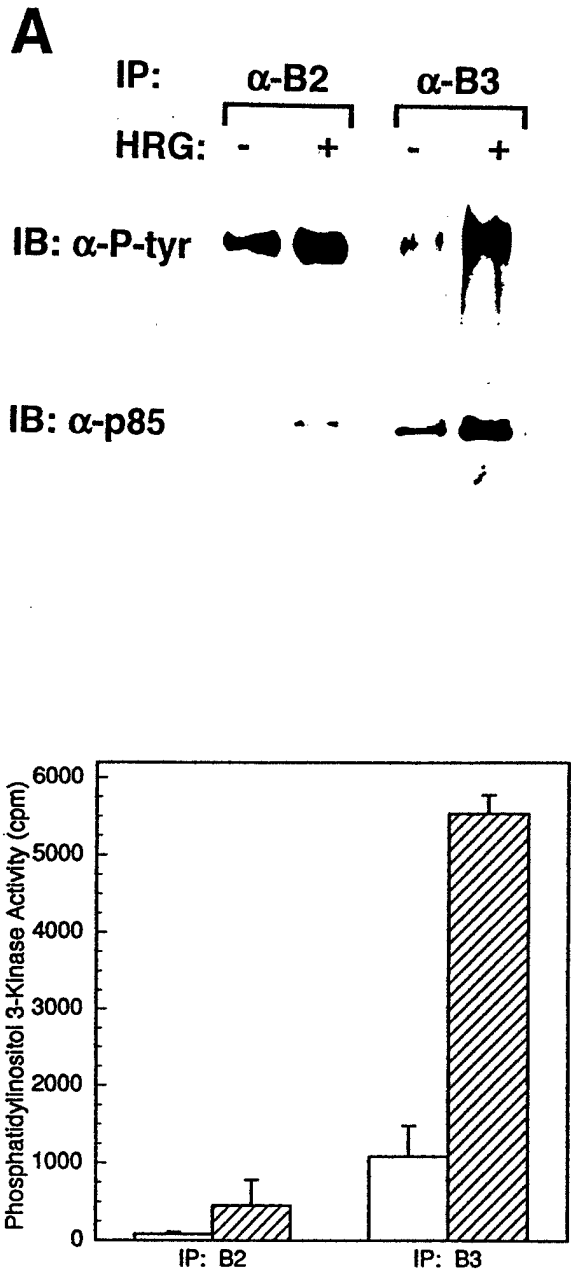

FIG. 3. **Detection of the p85 subunit and catalytic activity of PI 3-kinase in ErbB2 and ErbB3 immunoprecipitates.** COS7 cells transfected with ErbB2 and ErbB3 expression plasmids were treated for 20 min in the presence of absence of 1 nM heregulin. Cells were lysed, and lysates immunoprecipitated with either ErbB2 (α–B2) or ErbB3 (α–B3) antibody. *A*, aliquots of immunoprecipitates were immunoblotted with antibodies recognizing phosphotyrosine (α–P-tyr) and p85 (α–p85). *B*, aliquots of immunoprecipitates were analyzed for PI 3-kinase activity. Shown are the averages of three independent experiments with error bars indicating the standard error.

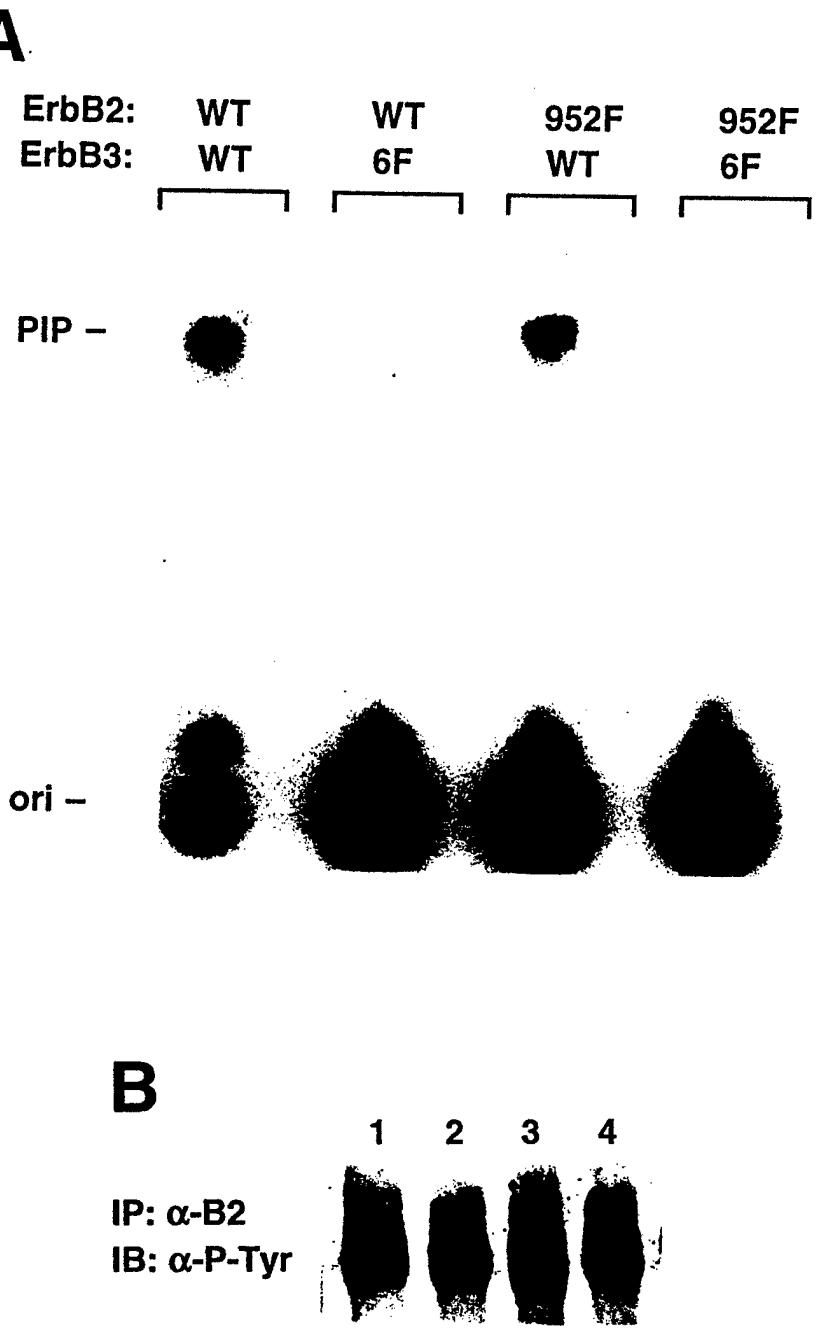

FIG. 4. **PI 3-kinase catalytic activity in ErbB2 immunoprecipitates from cells expressing wild-type or mutant ErbB receptors.** COS7 cells transfected with the indicated ErbB2 or ErbB3 expression plasmids were treated for 20 min with 1 nM heregulin. Cells were lysed, and lysates immunoprecipitated with ErbB2 antibody. *A*, aliquots of the immunoprecipitates were analyzed for PI 3-kinase activity by thin layer chromatography. *B*, aliquots of immunoprecipitates were immunoblotted with antibodies recognizing phosphotyrosine (lanes 1, ErbB2/ErbB3; 2, ErbB2/ErbB3-6F; 3, ErbB2-952F/ErbB3; 4, ErbB2-952F/ErbB3-6F). Results shown are representative of three independent experiments.

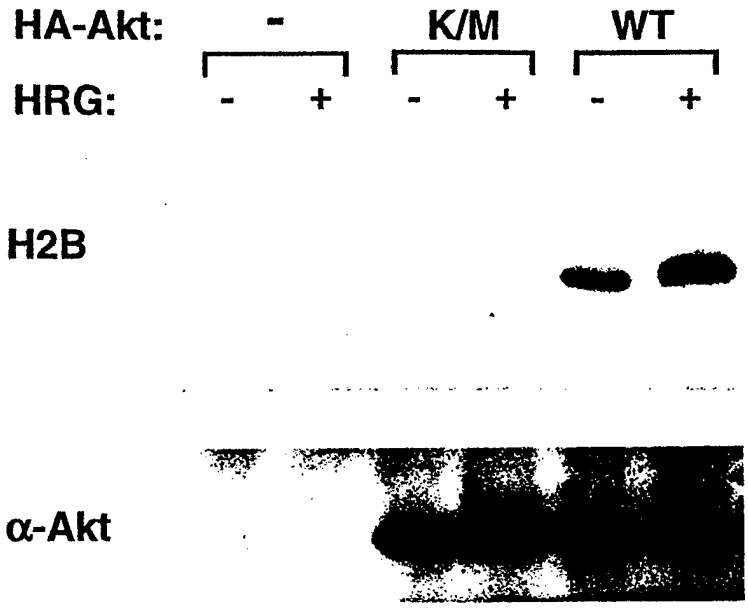

FIG. 5. **Heregulin-dependent Akt activation via ErbB2/ErbB3 co-receptors.** COS7 cells were transfected with ErbB2 and ErbB3 expression plasmids, along with the indicated HA-tagged Akt expression plasmids: pCMV5 vector control (—), Akt-K/M mutant (K/M), or wild-type Akt (WT). Cells were treated for 30 min in the presence or absence of 1 nM heregulin, lysed, and lysates immunoprecipitated with hemagglutinin (HA) antibody. Immunoprecipitates were analyzed for Akt activity by *in vitro* protein kinase assays employing histone 2B (H2B) as a substrate, and immunoprecipitated HA-Akt was detected by immunoblotting with an Akt-specific antibody (α-Akt). These results are representative of three independent experiments.

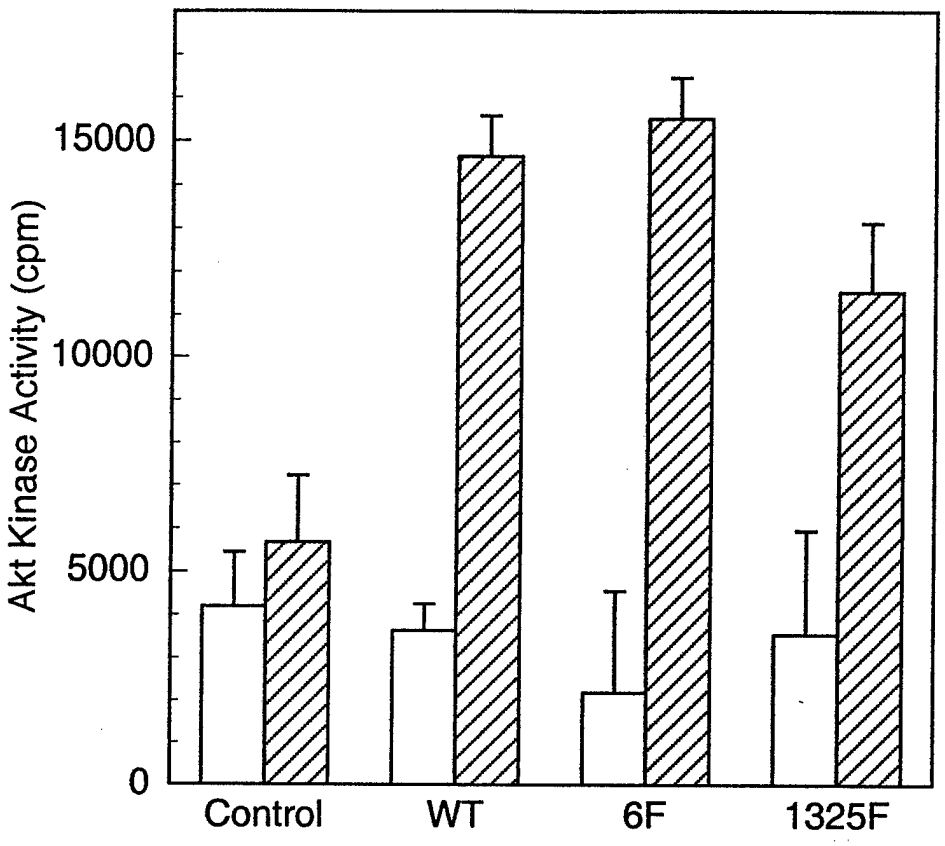

FIG. 6. **Heregulin-dependent Akt activation in cells transfected with wild-type or mutant ErbB3 receptors.** COS7 cells were transiently cotransfected with a pcDNA3 vector incorporating the ErbB2 cDNA and either the parent expression vector (-) or vector incorporating either wild-type ErbB3 (WT), ErbB3-6F (6F), or ErbB3-1325F (1325F) cDNA as indicated. Transfected cells were treated for 30 min in the absence (open bars) or presence (hatched bars) of 1 nM heregulin and subjected to detergent lysis. Cell lysates were immunoprecipitated with an Akt antibody, and immunoprecipitates analyzed for Akt activity by *in vitro* protein kinase assays employing an Akt-specific peptide substrate. Shown are the averages of three independent experiments with error bars indicating the standard error.

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APPENDICES

Manuscripts published in the funding period:

The following published manuscripts resulted directly from work performed during the four-year funding period. Copies of each are appended to the progress report.

1. Hellyer, N.J., Kim, H.-H., Greaves, C.H., Sierke, S.L., and Koland, J.G. (1995) "Cloning of the Rat *ErbB3* cDNA and Characterization of the Recombinant Protein," *Gene* **165**, 279-284.
3. Sierke, S.L., Cheng, K., Kim, H.-H., and Koland, J.G. (1997) "Biochemical Characterization of the Protein Tyrosine Kinase Homology Domain of the ErbB3 (HER3) Receptor Protein," *Biochem. J.*, **322**, 757-763.
4. Hellyer, N.J., Cheng, K., and Koland, J.G. (1998) "ErbB3 (HER3) Interaction with the p85 Regulatory Subunit of Phosphoinositide 3-Kinase," *Biochem. J.* **333**, 757-763.
5. Kim, H.-H., Vijapurkar, U., Hellyer, N.J., Bravo, D., and Koland, J.G. (1998) "Signal Transduction by Epidermal Growth Factor and Heregulin via the Kinase-deficient ErbB3 Protein," *Biochem. J.* **334**, 189-195.
6. Vijapurkar, U., Cheng, K., and Koland, J.G. (1998) "Mutation of a Shc Binding Site Tyrosine Residue in ErbB3/HER3 Blocks Heregulin-dependent Activation of Mitogen-activated Protein Kinase," *J. Biol. Chem.* **273**, 20996-21002.

Abstracts presented in the four-year funding period:

1. Kim, H.-H., Hellyer, N.J., Sierke, S.L., and Koland J.G. (1995) "Epidermal Growth Factor-stimulated Phosphorylation of the Kinase-deficient ErbB3 Protein," ASBMB, San Francisco.
2. Hellyer, N.J., Kim, H.-H., Greaves, C.H., Sierke, S.L., and Koland, J.G. (1995) "Molecular Cloning of the Rat ErbB3 cDNA and Characterization of the Gene Product," ASBMB, San Francisco.
3. Shearer, M.C., Kim, H.-H., Kratz, D.A., and Koland, J.G. (1996) "Heregulin-dependent Activation of MAP Kinases in Cultured Rat Astrocytes," ASBMB, New Orleans.
4. Hellyer, N.J., and Koland, J.G. (1996) "Characterizing the Interaction between ErbB3/HER3 and the p85 Subunit of Phosphatidylinositol 3-Kinase with the Yeast Two-Hybrid System," ASBMB, New Orleans.

5. Vijapurkar, U., and Koland, J.G. (1997) "Mutation of a Shc Binding Site Tyrosine Residue in ErbB3/HER3 Blocks Heregulin-dependent Activation of Mitogen-activated Protein Kinase," ASBMB, San Francisco.

6. Koland, J.G. and Vijapurkar, U.P. (1997) "Signal Transduction by the ErbB3/HER3 Heregulin Receptor," Department of Defense Breast Cancer Research Program Meeting, Washington, DC.

Personnel receiving pay from the negotiated effort:

1.	John G. Koland, Ph.D.	Principal Investigator
2.	Deborah Kratz, B.S.	Research Assistant II
3.	Dolores Bravo, M.S.	Research Assistant II

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Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein

Susan L. SIERKE, Kunrong CHENG, Hong-Hee KIM* and John G. KOLAND†
Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA 52242, U.S.A.

The putative protein tyrosine kinase domain (TKD) of the ErbB3 (HER3) receptor protein was generated as a histidine-tagged recombinant protein (hisTKD-B3) and characterized enzymologically. CD spectroscopy indicated that the hisTKD-B3 protein assumed a native conformation with a secondary structure similar to that of the epidermal growth factor (EGF) receptor TKD. However, when compared with the EGF receptor-derived protein, hisTKD-B3 exhibited negligible intrinsic protein tyrosine kinase activity. Immune complex kinase assays of full-length ErbB3 proteins also yielded no evidence of catalytic activity. A fluorescence assay previously used to characterize the nucleotide-binding properties of the EGF receptor indicated that the ErbB3 protein was unable to bind nucleotide. The hisTKD-B3 protein was subsequently found to be an excellent substrate for the EGF receptor protein tyrosine kinase, which suggested that *in vivo* phosphorylation of ErbB3 in response to EGF could be attributed to a direct cross-phosphorylation by the EGF receptor protein tyrosine kinase.

INTRODUCTION

Discovered by molecular cloning [1,2], the *ErbB3* gene encodes a member of the ErbB subfamily of receptor protein tyrosine kinases [3]. Like the prototypical epidermal growth factor (EGF) receptor, the ErbB3 protein is predicted to consist of an extracellular ligand-binding domain, a transmembrane domain, an intracellular protein tyrosine kinase domain (TKD) and a C-terminal phosphorylation domain. Despite its structural similarity to other ErbB family receptors (EGF receptor, ErbB2/Neu, ErbB4), the presence of protein tyrosine kinase activity in ErbB3 has been questioned [2], as the deduced amino acid sequence of the protein shows three substitutions for residues invariantly conserved in all protein tyrosine kinases with known sequence [4]. Efforts to resolve this question have led to conflicting results. Two groups have detected ligand-stimulated protein tyrosine kinase activity in a chimaeric EGF receptor/ErbB3 protein, and concluded that the ErbB3 cytosolic domain possesses intrinsic catalytic activity [5,6]. However, a third group found negligible protein kinase activity in a recombinant bovine ErbB3 protein [7].

Recently, the ErbB3 protein has been shown to bind EGF-related polypeptides in the neuregulin (heregulin) family [8–10]. In cultured cells expressing ErbB3, the protein has been seen to be phosphorylated on tyrosine residues in response to EGF or neuregulin [11–13]. As this phosphorylation is dependent on the co-expression of either the EGF receptor or ErbB2 [14–21], it has been considered that the ErbB3 protein may be a physiological substrate for the protein tyrosine kinase activities of the EGF receptor and ErbB2. Indeed it appears that the ErbB3 protein may form receptor heterodimers with either the EGF receptor or ErbB2 protein (reviewed in [22–24]). The role of any intrinsic protein tyrosine kinase activity of ErbB3 in the phosphorylation of ErbB3 and its associated ErbB family members within the context of receptor heterodimers remains unclear.

In order to assess the catalytic potential of ErbB3, the cytosolic domain of the protein and that of the well-characterized EGF receptor were generated by use of the baculovirus/insect cell expression system. The purified recombinant proteins were characterized by CD spectroscopy, protein tyrosine kinase activity assays and a recently described nucleotide-binding assay [25]. The recombinant ErbB3 protein was seen to be devoid of intrinsic protein tyrosine kinase activity, and indeed appeared unable to bind nucleotide. The ErbB3 cytosolic domain was subsequently found to be an excellent substrate for the EGF receptor protein tyrosine kinase. Together these results indicated that the observed phosphorylation of ErbB3 in the cellular context might be effected by the protein tyrosine kinase activities of other ErbB family members.

EXPERIMENTAL

Cell lines and reagents

All cell lines were purchased from American Type Culture Collection and cultured as recommended. 2′(3′)-*O*-(2,4,6-Trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) was obtained from Molecular Probes. [γ-^{32}P]ATP ($\sim$ 3000 Ci/mmol) was supplied by Dupont–New England Nuclear. ErbB3-specific (2F12) and EGF-receptor-specific (LA1) monoclonal antibodies were purchased from NeoMarkers and Upstate Biotechnology respectively. Phosphotyrosine-specific monoclonal antibody (PY20) was obtained from Leinco Technologies. Horseradish

Abbreviations used: EGF, epidermal growth factor; TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; TKD, protein tyrosine kinase domain; hisTKD61, C-terminally complete EGF receptor cytosolic domain protein; hisTKD38, C-terminally truncated EGF receptor cytosolic domain; hisTKD-B3, ErbB3 cytosolic domain protein.

* Present address: Tumor Immunology, Dana Farber Cancer Institute, Department of Medicine, Harvard Medical School, 44 Binney Street, Boston, MA 02115, U.S.A.

† To whom correspondence should be addressed.

peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were purchased from Amersham. The fusion protein GST–TK7 [26] and NIH-3T3 cells expressing the rat ErbB3 cDNA [27] have been previously described.

Expression of EGF receptor and ErbB3 TKD forms

Recombinant human EGF receptor and rat ErbB3 TKD forms, each with a Met-His-His-His-His-His-His leader peptide, were expressed with the baculovirus/insect cell system. The hisTKD38-coding sequence was generated from the EGF receptor cDNA in pMMTV-ER [28] by PCR with the primers 5′-TGCTCTAGACCATGCACCACCACCACCACCACCGA-AGGCGCCACATCGTTCGG-3′ (forward) and 5′-CCCCCG-GGCTAGTTGGAGTCTGTAGGACTTGGCAA-3′ (reverse). The forward primer included an *Xba*I restriction site, a start codon (underlined) and six His codons, as well as the coding sequence for amino acid residues 645–651 of the EGF receptor. The reverse primer was complementary to the coding sequence for residues 965–972 of the EGF receptor, and introduced a stop codon (underlined) and a *Sma*I restriction site into the PCR product. The resulting PCR product was subcloned into the baculovirus transfer vector pAcYMP1 [29] to yield pAc-TKD38. A baculovirus transfer vector for hisTKD61 (pAc-TKD61) was generated by cloning a cDNA fragment encoding the EGF receptor C-terminus into pAc-TKD38.

The coding sequence for the rat ErbB3 TKD was amplified by PCR from a previously characterized rat ErbB3 cDNA clone, pBS-rB3 [27]. The forward primer, 5′-TGCTCTAGACCATG-CACCACCACCACCACCACCGAATTCGGATTCAGAA-CAAAAGGGCTA-3′, included an *Xba*I site, a start codon (underlined), six His codons and the codons for amino acid residues 668–674 of ErbB3. The reverse primer, 5′-ACAA-GCTGCAGAGATGAC-3′, was complementary to a coding sequence within the rat ErbB3 cDNA downstream of a unique *Nde*I restriction site. The resulting PCR product was cloned into pBS-rB3 to yield a cDNA encoding the hisTKD-B3 protein, which was then subcloned into pAcYMP1. The authenticity of the PCR-amplified sequences present in each transfer vector was directly verified by DNA sequencing.

The purified baculovirus transfer vectors were co-transfected with BaculoGold baculovirus DNA (Pharmingen) into cultured Sf21 cells [30]. Recombinant baculovirus clones were isolated by an end point dilution method [31], and viral clones expressing high levels of the recombinant TKDs were identified by immunoblotting lysates of virally infected Sf21 cells.

For large-scale preparation of recombinant proteins, Sf21 cells were grown in spinner flask culture (125 ml) to a density of (1–2) $\times 10^6$ cells/ml, then infected with recombinant virus (~ 10 plaque-forming units/cell) [30]. At 48 h after infection, cells were harvested and washed gently in 20 ml of insect cell lysis buffer (20 mM Tris/HCl, 0.5 M NaCl, 5 mM imidazole, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, pH 7.9) supplemented with 250 mM sucrose. The cells were resuspended in 10 ml of sucrose-free lysis buffer and sonicated. The homogenate was clarified by centrifugation for 20 min at 80000 ***g***, and supplemented with Triton X-100 to a final concentration of 0.05%. The solution was applied to a 5 ml iminodiacetic acid–Sepharose 6B column (Sigma) that had been charged with 50 mM nickel sulphate and equilibrated with binding buffer (20 mM Tris/HCl, 0.5 M NaCl, 0.05% Triton X-100, pH 7.9) supplemented with 5 mM imidazole. The column was washed with ten column volumes of binding buffer (5 mM imidazole) and six column volumes of binding buffer supplemented with 60 mM imidazole, then eluted with binding buffer supplemented with 250 mM imidazole. Peak fractions in the eluate were identified by protein assays [32] and pooled. Free imidazole was removed by extensive dialysis against TKD dialysis buffer [20 mM Tris/HCl, 100 mM NaCl, 10% (v/v) glycerol, 0.05% Triton X-100, pH 7.9]. The purified TKD forms ($\sim 95\%$ pure, typically 1–2 mg of total protein) were supplemented with dithiothreitol to 1 mM and glycerol to 45% (v/v) and stored at −20 °C. All purification steps were carried out at 4 °C or on ice. In protein purifications for CD measurements, Triton X-100 was omitted from the column elution and final dialysis buffers.

In vitro protein tyrosine kinase assays

TKD proteins (0.25 μM) were incubated for 5 min at room temperature in TKD dialysis buffer (36 μl total volume) with 15 μM [γ-^{32}P]ATP ($\sim 10^4$ c.p.m./pmol), 10 mM $MnCl_2$ or $MgCl_2$, and 0.1% Triton X-100 added to the indicated concentrations. After quenching of the reactions by the addition of SDS/PAGE sample buffer, phosphoproteins were resolved by SDS/PAGE [33] and detected by autoradiography. Assays of exogenous peptide-phosphorylation activity included GST–TK7 (5 μg), a glutathione S-transferase fusion protein incorporating residues 943–1011 of the EGF receptor protein that has previously been shown to be an excellent protein tyrosine kinase substrate [26].

The cross-phosphorylation of the ErbB3 TKD (hisTKD-B3) by the truncated EGF receptor TKD (hisTKD38) was assayed as described above, except that the incubations were carried out for 15 min at room temperature. Phosphoproteins were then identified either by immunoblotting with the phosphotyrosine-specific antibody PY20 or by autoradiography. The kinetics of the cross-phosphorylation reaction were assayed by incubating hisTKD38 (0.25 μM) in the presence of 10 mM $MnCl_2$, 15 μM [γ-^{32}P]ATP and various concentrations (0–2.5 μM) of hisTKD-B3 for 5 min at room temperature in TKD dialysis buffer. The final glycerol concentrations of the samples were adjusted to a constant 30% (v/v). The ^{32}P-labelled phosphoproteins were resolved by SDS/PAGE, identified by autoradiography, and quantified by scintillation counting of bands excised from dried gels. V_{max} and K_m were determined by the fitting of rate equations with a non-linear least-squares minimization alogrithm [34], and the hyperbolic curve generated is shown in Figure 6(B).

In immune complex kinase assays, immunoprecipitates were incubated with [γ-^{32}P]ATP ($\sim 10^4$ c.p.m./pmol) for 10 min at room temperature. Reactions contained 40 mM Hepes/Na (pH 7.4), 0.05% Triton X-100, 10 mM $MnCl_2$, or 10 mM $MgCl_2$ and 3 mM $MnCl_2$, and 17 μM ATP. Then 5 × SDS/PAGE sample buffer was added to stop the reactions, and the samples were subjected to electrophoresis and autoradiography.

Fluorescence spectroscopic analysis of nucleotide binding

Binding of the TNP-ATP nucleotide analogue to the recombinant TKDs was analysed by a recently described fluorescence assay [25]. Briefly, fixed concentrations of recombinant protein were titrated with increasing concentrations of TNP–ATP (0–7.5 μM) as the fluorescence of the nucleotide was recorded. Fluorescence titration data were corrected for the contribution of both free and non-specifically bound TNP-ATP, as determined by titrations performed with the inclusion of excess ATP, and for inner filter quenching effects observed at high TNP-ATP concentrations. Dissociation constants for TNP-ATP binding were subsequently determined by fitting of a theoretical binding equation to the titration data [25].

CD spectroscopic measurements

UV CD spectra of recombinant proteins were recorded with an Aviv 62DS instrument with solutions of 2 μM protein in 10 mM Tris/HCl/50 mM NaCl/25% (v/v) glycerol, pH 7.9, held in 2 mm cells thermostatically controlled at 4 °C. A solvent blank spectrum was subtracted from each protein spectrum. Analysis of CD spectra for determination of the content of secondary-structural elements was carried out with the aid of spectral decomposition software [35].

RESULTS

Generation and characterization of recombinant EGF receptor and ErbB3 TKDs

In order to compare the catalytic properties of the EGF receptor and the ErbB3 protein, the TKDs of these receptors were expressed as recombinant proteins with the baculovirus/insect cell system. Baculovirus expression vectors for two distinct EGF receptor TKD forms, one with an authentic C-terminus (hisTKD61) and one with a highly truncated C-terminus (hisTKD38), and a full-length ErbB3 TKD form (hisTKD-B3) were constructed (Figure 1). The three recombinant TKDs were expressed in Sf21 cells, and each of these proteins was effectively purified by Ni^{2+}-chelating column chromatography (Figure 2A).

The secondary structures of the purified recombinant proteins were analysed by CD spectroscopy. The spectra of the C-terminally complete hisTKD61 and hisTKD-B3 proteins were qualitatively similar, and spectral decomposition analysis [35] indicated similar contents of α-helix, β-sheet, β-turn and random structural elements (Figure 2B). Given that the EGF receptor-derived hisTKD61 protein was found to possess a catalytic activity comparable with that of the native EGF receptor protein (results not shown), it was assumed that this recombinant protein was folded in a native conformation. The similarity of the CD spectrum of the hisTKD-B3 protein to that of the hisTKD61 protein then suggested that the ErbB3-derived protein also assumed a native conformation.

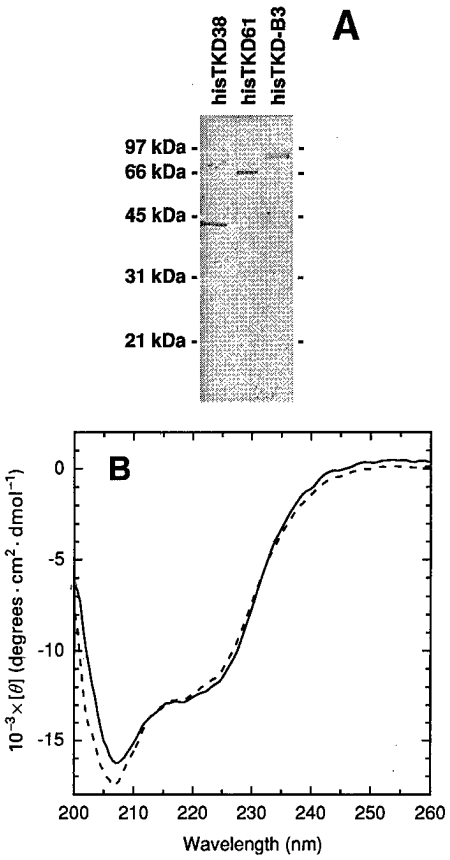

Figure 2 Characterization of EGF receptor and ErbB3 TKDs by SDS/PAGE and CD spectroscopy

(**A**) Recombinant TKD proteins generated with the baculovirus/insect cell system were purified to near-homogeneity by Ni^{2+}-chelating column chromatography (see the Experimental section). SDS/PAGE analysis with silver staining of 0.5 μg samples of the three TKD forms is shown. (**B**) CD spectra of EGF receptor (——) and ErbB3 (– – –) TKDs were recorded and analysed for context of secondary-structural elements (see the Experimental section). Percentages of α-helix, β-sheet, β-turn and random elements were 41, 35, 10 and 14% respectively for the hisTKD61 protein, and 40, 38, 9 and 13% respectively for the hisTKD-B3 protein.

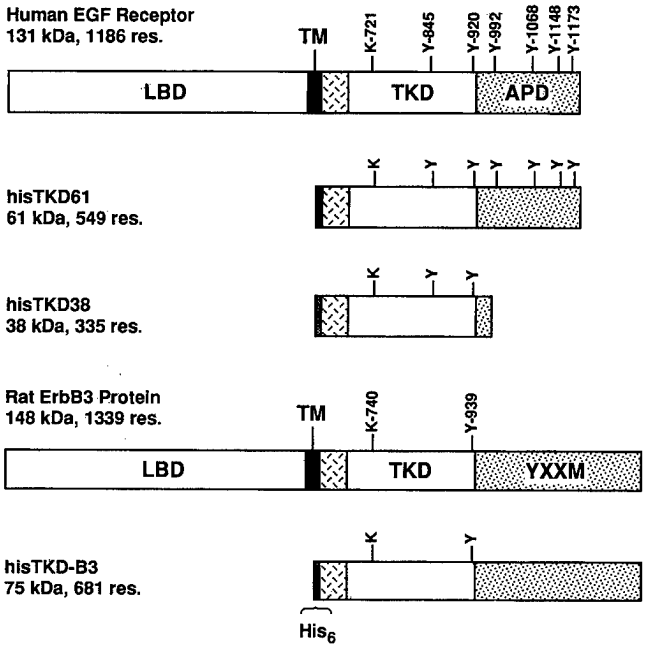

Figure 1 Schematic structures of recombinant EGF and ErbB3 receptor TKDs

The putative functional domains of the two receptor proteins are indicated: ligand-binding domain (LBD), transmembrane domain (TM), protein tyrosine kinase domain (TKD) and autophosphorylation domain (APD). Candidate phosphorylation sites in the EGF receptor are identified, and YXXM labels the C-terminal domain of ErbB3 that contains seven repetitions of the consensus phosphatidylinositol 3-kinase-binding site, Tyr-Xaa-Xaa-Met. The Met-His-His-His-His-His-His leader peptide introduced into each of the recombinant proteins is also indicated (His_6).

Catalytic activities of recombinant EGF receptor and ErbB3 TKDs

Previous studies of a full-length EGF receptor TKD expressed in the baculovirus/insect cell system indicated that the hisTKD61 protein would be an active protein tyrosine kinase showing selectivity for Mn^{2+} over Mg^{2+} as an activating metal ion [36,37]. The hisTKD38 protein was also expected to be fully active, although it was expected that this truncated protein would lack the strong autophosphorylation activity of the full-length TKD. The autophosphorylation and substrate phosphorylation activities of the two recombinant EGF receptor TKD forms were compared with those of the ErbB3-derived protein (Figure 3).

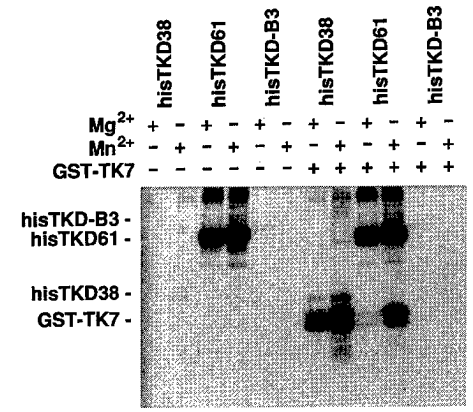

Figure 3 Autophosphorylation and substrate-phosphorylation activities of the EGF receptor and ErbB3 TKDs

Each of the TKD proteins (0.25 μM) was incubated for 5 min at room temperature in the presence of 15 μM [γ-^{32}P]ATP, and either 10 mM $MgCl_2$ or 10 mM $MnCl_2$ as indicated. TKD autophosphorylation was analysed by SDS/PAGE and autoradiography. Substrate phosphorylation activities of the TKD proteins were similarly assayed with the inclusion of 5 μg of the protein substrate GST–TK7 in the incubation as indicated. The GST–TK7 protein shows multiple bands when phosphorylated.

These experiments employed a recombinant fusion protein (GST–TK7) known to be a substrate for the EGF receptor and c-Src protein tyrosine kinases [26], and both Mg^{2+} and Mn^{2+} were tested as activators of the phosphorylation reactions.

Whereas the hisTKD61 protein showed a strong autophosphorylation, autophosphorylation of the hisTKD38 and hisTKD-B3 proteins was much weaker. Phosphoamino acid analyses (results not shown) indicated that, whereas the weak autophosphorylation of the hisTKD38 protein corresponded to the incorporation of phosphotyrosine, the hisTKD-B3 protein was not phosphorylated on tyrosine residues (see also Figure 6A). Each of the three TKD forms was phosphorylated to a very small extent on serine and threonine, which was apparently due to a contaminating serine/threonine kinase activity. Significantly, the substrate-phosphorylation activity of the hisTKD-B3 protein was negligible compared with that of the EGF receptor-derived TKDs. Several other attempts to detect protein tyrosine kinase activity in the ErbB3 TKD also yielded negative results. For example, when a distinct ErbB3 TKD lacking the hexa-His leader peptide was generated with a vaccinia virus expression system, intrinsic protein tyrosine kinase activity was again not evident (results not shown).

Catalytic activity of the full-length ErbB3 protein *in vitro*

The protein tyrosine kinase activity of the full-length ErbB3 protein was also assessed. Here, the native ErbB3 protein was immunoprecipitated from cells expressing the protein at a high level either as a consequence of gene transfection (3T3-B3 cells) or tumorigenesis (MDA-MB-453 and SK-BR-3) (Figure 4A). For comparison, the EGF receptor was immunoprecipitated from MDA-MB-468 cells. Immunoprecipitated proteins were incubated with [γ-^{32}P]ATP and bivalent metal ions. As expected, EGF receptor immune complexes showed strong autophosphorylation. In contrast, ErbB3 immunoprecipitates exhibited negligible autophosphorylation activity (Figure 4B). Neither varying the assay conditions nor stimulating with the ligand neuregulin led to the detection of ErbB3 kinase activity (results not shown). Both rat and human ErbB3 proteins were tested here, as the transfected NIH-3T3 cells expressed the rat ErbB3 protein and the cancer cell lines used were derived from human breast carcinomas.

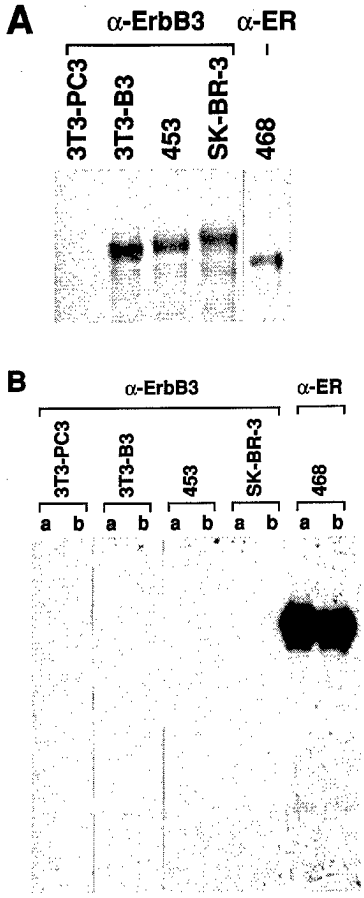

Figure 4 Autophosphorylation activities of full-length EGF receptor and ErbB3 proteins

(**A**) Immunoprecipitation of ErbB3 and EGF receptor from cultured human breast cancer cell lines and NIH-3T3 cells expressing recombinant rat ErbB3 protein. Detergent lysates were prepared from NIH-3T3 fibroblasts transfected with the parent pcDNA3 expression vector (3T3-PC3) or the pcDNA3-ErbB3 vector (3T3-B3) and three human breast cancer cell lines: MDA-MB-453 (453), MDA-MB-468 (468) and SK-BR-3. Aliquots of each lysate (4 mg of protein) were precleared with Protein G–agarose, then immunoprecipitated with either ErbB3-specific antibody 2F12 (α-ErbB3) or EGF-receptor-specific antibody LA1 (α-ER) as indicated. After two washes, precipitates were resuspended and one-half of each sample was analysed by SDS/PAGE and immunoblotting with the immunoprecipitating antibodies. (**B**) Immune complex kinase assays performed with ErbB3 and EGF receptor immunoprecipitates. One-fifth of each suspended immunoprecipitate analysed in (**A**) was incubated for 10 min at room temperature in the presence of 17 μM [γ-^{32}P]ATP and either 10 mM $MnCl_2$ (lanes a) or a mixture of 10 mM $MgCl_2$ and 3 mM $MnCl_2$ (lanes b). The phosphoproteins were resolved by SDS/PAGE and identified by autoradiography.

Nucleotide-binding properties of EGF receptor and ErbB3 TKD proteins

Previously, we have shown that the fluorescent nucleotide analogue TNP–ATP binds to recombinant EGF receptor TKD forms, and that this binding can be conveniently monitored by measuring the enhancement of TNP-ATP fluorescence that occurs on binding to the TKD [25]. The Mn·TNP-ATP complex was found to be a functional substrate for the EGF receptor protein tyrosine kinase, which apparently mimics the authentic substrate Mn·ATP. The TNP-ATP nucleotide binding exhibited

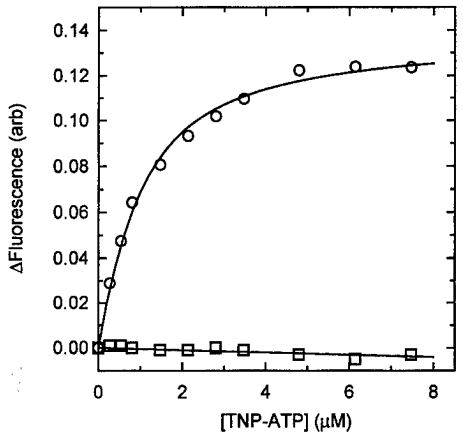

Figure 5 Nucleotide-binding properties of recombinant EGF receptor and ErbB3 TKDs

The interaction of the nucleotide analogue TNP-ATP with the recombinant TKD proteins was analysed by fluorescence spectroscopy as previously described [25]. Whereas the EGF-receptor-derived hisTKD61 protein (○) showed a high-affinity interaction with TNP-ATP ($K_d = 0.75 \pm 0.24$ μM), the ErbB3-derived hisTKD-B3 protein (□) showed no interaction.

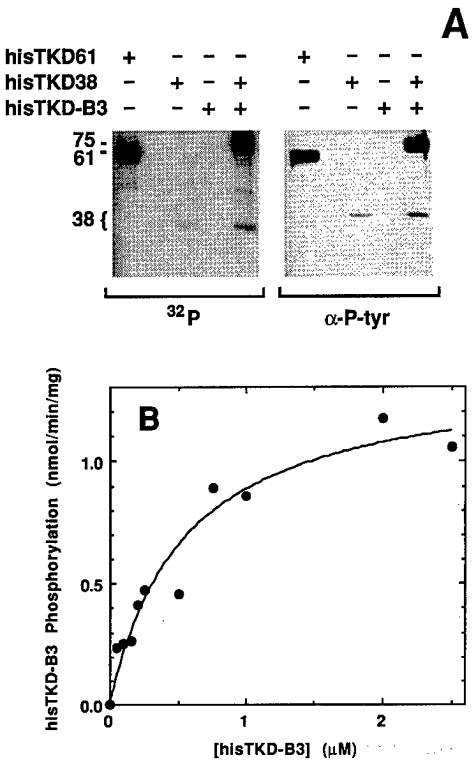

Figure 6 Autophosphorylation and cross-phosphorylation activities of EGF receptor and ErbB3 TKDs

(**A**) The C-terminally complete EGF receptor TKD (hisTKD61), the truncated EGF receptor TKD (hisTKD38) and the ErbB3 TKD (hisTKD-B3) (see Figure 1) (each at 0.25 μM concentration) were incubated either separately or together as indicated for 15 min at room temperature in the presence of 10 mM $MnCl_2$ and either 15 μM [γ-^{32}P]ATP (left panel) or 15 μM ATP (right panel). Phosphorylation was analysed by SDS/PAGE and autoradiography (left panel) or by anti-phosphotyrosine immunoblotting (right panel). (**B**) The kinetics of phosphorylation of hisTKD-B3 by hisTKD38 were analysed by incubating hisTKD38 (0.25 μM) in the presence of 0–2.5 μM hisTKD-B3 for 5 min at room temperature as in (**A**). ErbB3 phosphorylation was assayed by SDS/PAGE, autoradiography and scintillation counting of excised gel bands. The hyperbolic curve best fitting the experimental data is shown ($K_m = 0.54$ μM; $V_{max} = 1.4$ nmol/min per mg).

by the EGF-receptor-derived hisTKD61 protein was directly compared with that of the hisTKD-B3 protein (see Figure 5). Whereas the hisTKD61 protein bound the nucleotide analogue with a dissociation constant in the micromolar range ($K_d = 0.75 \pm 0.24$ μM), there was no detectable interaction of the nucleotide analogue with the ErbB3-derived protein. Failure of the fluorescent nucleotide analogue to interact with hisTKD-B3 precluded attempts to address directly the ATP and Mn·ATP binding properties of this protein. However, the inability of the ErbB3 protein to bind the nucleotide analogue was certainly consistent with its observed lack of protein tyrosine kinase activity. In related studies (results not shown), a truncated ErbB3 TKD protein lacking the C-terminal phosphorylation domain was found to associate with TNP-ATP and ATP, but did not detectably interact with Mn·TNP-ATP and also showed no catalytic activity.

ErbB3 as a protein tyrosine kinase substrate

In our earlier work [38], C-terminal sequences of the ErbB3 receptor protein were found to be excellent substrates for the EGF receptor protein tyrosine kinase with K_m values ranging from 1 to 30 μM. This suggested that if the ErbB3 receptor was not itself an active protein kinase, it might serve as a substrate for another receptor protein kinase in the ErbB family. To examine the potential for EGF receptor/ErbB3 cross-phosphorylation, the EGF receptor-derived hisTKD38 protein was incubated with the ErbB3-derived hisTKD-B3 under phosphorylating conditions (Figure 6A). Whereas the hisTKD38 and hisTKD-B3 proteins alone showed negligible autophosphorylation activities when compared with the C-terminally complete hisTKD61 protein, hisTKD-B3 was strongly phosphorylated on incubation with hisTKD38. This phosphorylation could be detected by either autoradiography of ^{32}P-labelled proteins or immunoblotting with anti-phosphotyrosine (Figure 6A). The K_m and V_{max} for phosphorylation of the hisTKD-B3 substrate by the hisTKD38 protein kinase were approx. 0.5 μM and 1.4 nmol/min per mg respectively (Figure 6B). Hence the hisTKD-B3 protein exhibited a K_m value among the lowest documented for substrates for the EGF receptor protein tyrosine kinase. The hisTKD-B3 protein was also efficiently phosphorylated by the C-terminally complete EGF receptor protein kinase, hisTKD61 (results not shown), although the similar SDS/PAGE mobilities of the hisTKD-B3 and hisTKD61 proteins precluded a quantitative analysis of this phosphorylation reaction.

DISCUSSION

The *ErbB3* gene product has been predicted to be a receptor protein tyrosine kinase similar in structure and function to other EGF receptor family members [1,2]. We have attempted to detect intrinsic protein tyrosine kinase activity in the ErbB3 protein by various approaches. As the protein tyrosine kinase domains of a variety of other receptors have been produced in catalytically active form with the baculovirus system [29,39,40–42], we used this sytem in the generation of an ErbB3 cytosolic domain protein (hisTKD-B3). CD spectroscopic measurements indicated that the hisTKD-B3 protein was folded in a conformation similar to that of the corresponding EGF receptor cytosolic domain (hisTKD61), which displayed robust catalytic activity. However, the recombinant ErbB3 protein exhibited negligible catalytic activity under the same experimental conditions (Figure

3). Immune complex kinase assays of full-length ErbB3 proteins also failed to demonstrate intrinsic kinase activity (Figure 4). These results led to the conclusion that the ErbB3 protein is not intrinsically a protein kinase.

The potential of the ErbB3 protein to bind nucleotide substrates was assessed with the aid of the fluorescent nucleotide analogue TNP-ATP, which has previously been used to characterize the nucleotide-binding properties of the EGF receptor TKD [25]. Whereas the EGF receptor-derived hisTKD61 protein again bound TNP-ATP with high affinity (Figure 5), there was no observed enhancement of TNP-ATP fluorescence in the presence of the hisTKD-B3 protein. Because there was no apparent interaction of the analgoue with hisTKD-B3, it was not possible to use this assay to investigate the potential interaction of ErbB3 with the authentic substrate Mn·ATP. In a previous study of recombinant bovine ErbB3 [7], the receptor protein was seen to be specifically labelled by 5′-*p*-fluorosulphonylbenzoyladenosine, although again no evidence for intrinsic kinase activity was obtained. Given that the ErbB3 cytosolic domain here did not interact with the analogue TNP-ATP and also showed no catalytic activity, it is reasonable to suspect that the ErbB3 protein may be unable to bind ATP in the same manner as other protein tyrosine kinases.

The apparent absence of catalytic activity and failure to bind nucleotide substrate might be explained by the occurrence of non-conservative amino acid substitutions in the putative protein tyrosine kinase domain of ErbB3. Specifically, the residues Cys-721, His-740 and Asn-815 in human ErbB3 [2] correspond to Ala, Glu and Asp respectively in all other known protein tyrosine kinases [4]. Sequencing of the rat ErbB3 cDNA has revealed an Asp residue corresponding to Asn-815 in human ErbB3 [27], which suggested that the rat ErbB3 protein, unlike human ErbB3, might possess kinase activity. However, neither rat nor human ErbB3 showed evidence of catalytic activity in this study (Figure 4).

A previous study of the bovine ErbB3 protein also yielded no indication of significant kinase activity [7]. However, an apparent intrinsic protein tyrosine kinase activity was detected in other investigations of the human ErbB3 protein [5,6]. In these latter studies, *in vitro* phosphorylation of ErbB3 in an immune complex [5] and EGF-stimulated *in vivo* phosphorylation of a chimaeric receptor consisting of the extracellular domain of EGF receptor and cytosolic domain of ErbB3 were demonstrated [5,6]. It is possible that this observed ErbB3 phosphorylation resulted from the action of an associated non-ErbB3 kinase. For example, the *in vivo* and *in vitro* phosphorylations of kinase-deficient mutant forms of the EGF receptor have been demonstrated [43,44], and an ectopically expressed kinase-deficient EGF receptor mutant was shown to be cross-phosphorylated by endogenous wild-type EGF receptors [45].

The ErbB3 protein has been shown to function with ErbB2/neu as a high-affinity coreceptor for the neuregulin (heregulin) peptides [8,10]. Also, the EGF-dependent phosphorylation of the ErbB3 protein in human cancer cells expressing high levels of both EGF receptor and ErbB3 has been documented [11,12]. A variety of recent evidence is consistent with a general model in which pairs of distinct ErbB family receptor proteins function as receptor heterodimers [23]. In this model, receptor heterodimerization provides a mechanism for diversifying the signal-transduction pathways activated by polypeptide growth factors in the EGF family. As specific phosphorylated tyrosine residues within the unique C-termini of the ErbB family members have been shown to function as docking sites for distinct signal-transducing proteins such as phospholipase C, phosphatidylinositol 3-kinase, Grb2 and Shc [46], receptor phosphorylation in the context of heterodimers is a critical event in ErbB family receptor signal transduction. If devoid of intrinsic protein tyrosine kinase activity, the ErBB3 protein would be phosphorylated only in association with other ErbB family receptor proteins. Our observation that the ErbB3 protein was an excellent substrate for the EGF receptor *in vitro* (Figure 6) is consistent with the assumption that the documented *in vivo* phosphorylation of ErbB3 in response to either EGF or neuregulin results directly from the action of other ErbB family protein tyrosine kinases.

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Cloning of the rat *ErbB3* cDNA and characterization of the recombinant protein

(HER3; protein tyrosine kinase; Src homology domain; heregulin; oncogene)

Nathan J. Hellyer, Hong-Hee Kim, Charles H. Greaves, Susan L. Sierke and John G. Koland

Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA 52242-1109, USA

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SUMMARY

Three cDNA fragments that encoded all but the extreme N terminus of the rat ErbB3 protein were cloned by low-stringency screening of a rat liver cDNA library with a human *ERBB3* probe. The remaining 5'-end of the cDNA was generated by a reverse transcription-polymerase chain reaction method, and a single full-length rat *ErbB3* cDNA was assembled. A comparison of the deduced amino acid (aa) sequences of human and rat ErbB3 was made, and the effects of certain aa substitutions in the putative protein tyrosine kinase domain were considered. The rat *ErbB3* cDNA was subsequently expressed in cultured NIH-3T3 mouse fibroblasts, in which a high level of approx. 180-kDa recombinant ErbB3 (re-ErbB3) was generated. The rat re-ErbB3 produced in transfected fibroblasts was responsive to the polypeptide, heregulin, a known ligand for ErbB3. Challenge of transfected fibroblasts with heregulin stimulated the phosphorylation of rat re-ErbB3 on Tyr residues and promoted its association with the p85 subunit of phosphatidylinositol 3-kinase. Together, these results indicate that a fully functional rat *ErbB3* cDNA has been isolated, and that fibroblast cells expressing this cDNA will be suitable for investigations of the signal transduction mechanism of ErbB3.

INTRODUCTION

The *ERBB* (*HER*) family of human genes has four identified members. The prototypical c-*ERBB* (*HER*) gene encodes the well studied epidermal growth factor (EGF) receptor and was first identified as the cellular homolog of the v-*ErbB* oncogene of avian erythroblastosis virus (Downward et al., 1984). The EGF receptor consists of a single polypeptide chain that forms an extracellular growth factor binding domain, a short transmembrane (TM) domain, and an intracellular protein tyrosine kinase (PTK) domain. The *ERBB2* (*HER2*) gene, first characterized as a homolog of the rat *Neu* oncogene, also encodes a polypeptide growth factor receptor with PTK activity (Stern et al., 1986). The *ERBB3* (*HER3*) (Kraus et al., 1989; Plowman et al., 1990) and *ERBB4* (*HER4*) (Plowman et al., 1993a) genes have been more recently identified by molecular cloning. The predicted amino acid (aa) sequences of human ErbB3 and ErbB4 are closely related to those of the EGF receptor and ErbB2, which suggests that these two proteins are also receptors with intrinsic PTK activity.

Correspondence to: Dr. J.G. Koland, Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, IA 52242-1109, USA. Tel. (1-319) 335-6508; Fax (1-319) 335-8930; e-mail: nhellyer@blue.weeg.uiowa.edu

Abbreviations: aa, amino acid(s); Ab, antibody(ies); bp, base pair(s); EGF, epidermal growth factor; *ERBB3*/*ErbB3*, human/rat gene (DNA) encoding ErbB3 (also designated *HER3*); GRB2, growth factor receptor-bound protein 2; HRG, heregulin (Neu differentiation factor); oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; PI, phosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; PTK, protein tyrosine kinase(s); re-, recombinant; RT, reverse transcription(ase); SDS, sodium dodecyl sulfate; SH, Src homology domain; SHC, Src homology-2 α-collagen related protein; SLIC, single-strand ligation to ss-cDNA; ss, single strand(ed); STP, signal-transducing protein(s); TM, transmembrane; Xaa, any aa.

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A number of distinct polypeptide ligands that bind and activate ErbB family receptor proteins have now been identified. However, the relationships between the individual ligands and the four ErbB proteins are complex and not fully resolved. Whereas EGF and transforming growth factor α (TGFα) are identified ligands for the EGF receptor (Gill et al., 1987), a variety of polypeptides in the heregulin (HRG) family, which includes the heregulins (Holmes et al., 1992), Neu differentiation factor (NDF) (Wen et al., 1992), and the glial growth factors (Marchionni et al., 1993), have been characterized as ligands for ErbB3. Recent evidence indicates that the high-affinity HRG receptor is actually an ErbB2/ErbB3 heterodimer, with the ErbB3 protein being itself a low-affinity HRG receptor (Sliwkowski et al., 1994; Tzahar et al., 1994). In addition, the ErbB4 protein has been demonstrated to be a high-affinity receptor for HRG (Plowman et al., 1993b; Tzahar et al., 1994).

The intrinsic PTK activity of the ErbB proteins has been generally considered to be essential for their functions in the activation of mitogenic and developmental signal transduction pathways (Schlessinger and Ullrich, 1992). Although the predicted PTK domains of the ErbB proteins have aa sequences that agree well with the consensus sequence for identified PTK (Hanks and Quinn, 1991), human ErbB3 is unique in that three of the aa residues invariantly conserved in PTK are found altered in its predicted PTK domain sequence (Kraus et al., 1989; Plowman et al., 1990). This suggests that ErbB3 might not possess intrinsic PTK activity, and could have a cellular function quite distinct from those of the other ErbB family receptor proteins. To this end, several attempts have been made to demonstrate an intrinsic PTK activity in the *ERBB3* gene product.

Kraus et al. (1993) have documented the constitutive phosphorylation of ErbB3 that is endogenously produced in high levels in certain cultured human breast cancer cell lines. A ligand-dependent activation of the ErbB3 PTK domain in the context of a recombinant chimeric protein that incorporates the extracellular and membrane-spanning domains of the EGF receptor has also been demonstrated (Kraus et al., 1993; Prigent and Gullick, 1994). In contrast, our laboratory (H.-H. K., S.L.S. and J.G.K., data not shown) and others (Guy et al., 1994) have failed to detect intrinsic PTK activity in recombinant and native ErbB3 proteins. To enable the further examination of the catalytic and signal-transducing potentials of ErbB3, we have now isolated a full-length rat *ErbB3* cDNA and generated the corresponding protein in a cultured cell system by gene transfection. In this paper, we present an analysis of this full-length rat *ErbB3* coding sequence and a preliminary characterization of the rat re-ErbB3 protein.

```
MRATGTLQVLCFLLSLARGSEMGNSQAVCPGTLNGLSVTGDADNQYQTLYKLYEKCEVVM   60
   NDA   GL F          V                    E         R
GNLEIVLTGHNADLSFLQWIREVTAYVLVAMNEFSVLPLPNLRVVRGTQVYDGKFAIFVM  120
                          G     T
LNYNTNSSHALRQLKFTQLTEILSGGVYIEKNDKLCHMDTIDWRDIVRVRGAEIVVKNNG  180
              RL                                        D D     D
ANCPPCHEVCKGRCWGPGPDDCQILTKTICAPQCNGRCFGPNPNQCCHDECAGGCSGPQD  240
RS              SE   T          H
TDCFACRRFNDSGACVPRCPEPLVYNKLTFQLEPNPHTKYQYGGVCVASCPHNFVVDQTF  300
        H           Q                                        S
CVRACPPDKMEVDKHGLKMCEPCGGLCPKACEGTGSGSRYQTVDSSNIDGFVNCTKILGN  360
              N                    F
LDFLITGLTGDPWHKIPALDPEKLNVFRTVREITGYLNIQSWPPHMHNFSVFSNLTTIGG  420
        N
RSLYNRGFSLLIMKNLNVTSLGFRSLKEISAGRVYISANQQLCYHHSLNWTRLLRGPSEE  480
                                  I     R          KV    T
RLDIKYDRPLGECLAEGKVCDPLCSSGGCWGPAPGQCLSCRNYSREGVCVTHCNFLQGEP  540
     HN  RRD V                    G          G       N
REFVHEAQCFSCHPECLPMEGTSTYNGSGSDACARCAHFRDGPHCVNSCPHGILGAKGPI  600
   A  E        Q     A C     T  Q          S     V
YKYPDAQNECRPCHENCTQGCNGPELQDCLGQAEVLMSKPHLVIAVTV--GLAVILMILG  658
     V              K            TL  IG T  TM L  IA  V  F M
GSFLYWRGRRIQNKRAMRRYLERGESIEPLDPSEKANKVLARIFKETELRKLKVLGSGVF  718
 T
                 •                 •
                 A                 E
GTVHKGIWIPEGESIKIPVCIKVIEDKSGRQSFQAVTDHMLAVGSLDHAHIVRLLGLCPG  778
      V                                    I         •
                                                     D
SSLQLVTQYLPLGSLLDHVKQHRETLGPQLLLNWGVQIAKGMYYLEEHSMVHRDLALRNV  838
                          R  GA                      G   N  A
MLKSPSQVQVADFGVADLLPPDDKQLLHSEAKTPIKWMALESIHFGKYTHQSDVWSYGVT  898
L                           Y
VWELMTFGAEPYAGLRLAEIPDLLEKGERLAQPQICTIDVYMVMVKCWMIDENIRPTFKE  958
                            V
LANEFTRMARDPPRYLVIKRASGPGTPPAAEPSVLTTKELQEAELEPELDLDLDLEAEEE 1018
                       E    IA GP  HG  N K E V                D
GLA-TSLGSALSLPTGTLTRPRGSQSLLSPSSGYMPMNQSSLGEACLDSAVLGGREQFSR 1077
N  T T          V   N                    GN    S QE    S SS  RCP
PISLHPIPRGRPASESSEGHVTGSEAELQEKVSVCRSRSRSRSPRPRGDSAYHSQRHSLL 1137
 V    M   CL                        M
TPVTPLSPPGLEEEDGNGYVMPDTHLRGASSSREGTLSSVGLSSVLGTEEEDEDEEYEYM 1197
                V              K TP
NRKRRGSPPRPPRPGSLEELGYEYMDVGSDLSASLGSTQSCPLHPMAIVPSAGTTPDEDY 1257
  R  H   H    S                                  VP  M  T
EYMNRRRGAGGAGGDYAAMGACPAAEQGYEEMRAFQGPGHHAPHVRYARLKTLRSLEATD 1317
    Q DG  P                  S                  Q    H
SAFDNPDYWHSRLFPKANAQRT  1339
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Fig. 1. Deduced aa sequence of rat ErbB3. The rat *ErbB3* cDNA sequence encoded a protein of 1339 aa. Deviations in the human sequence (Kraus et al., 1989; Plowman et al., 1990) are listed below the rat sequence. The putative leader peptide and TM domain are indicated with bold underlines. The symbol (●) indicates three aa conserved in all characterized PTK that are substituted in the human ErbB3 sequence. Other identified sequence elements including several candidate binding sites for STP are indicated by underlining (see Table 1). The cDNA sequence has been deposited in GenBank/EMBL under accession No. U29339. **Methods:** A partial cDNA clone was isolated by screening a rat liver λZAPII library (Stratagene, La Jolla, CA, USA) at reduced stringency with a human *ERBB3* cDNA probe. Overlapping cDNAs were isolated by screening the same library at high stringency with restriction fragment probes derived from successively isolated rat *ErbB3* cDNAs. The remaining 5'-sequence of rat *ErbB3* cDNA was amplified by the 5'-SLIC/RT-PCR method (Dumas et al., 1991). First-strand cDNA synthesis was carried out with rat liver mRNA (1 μg) (Clontech, Palo Alto, CA, USA), Superscript RT (GIBCO-BRL, Grand Island, NY, USA) (10 units/μl), and random hexamer oligo primers (3.3 ng/μl). Four independent PCR products were cloned and sequenced. One product representing the consensus sequence was subcloned with the overlapping cDNA fragments to yield a full-length rat ErbB3 coding sequence.

EXPERIMENTAL AND DISCUSSION

(a) Cloning of the rat *ErbB3* cDNA

Screening of a rat liver bacteriophage λ cDNA library with a human *ERBB3* cDNA probe yielded several cDNA fragments. Sequence analyses indicated that four of these cDNA fragments (*1*) each had high similarity to

the human *ERBB3* cDNA, (*2*) were overlapping, and (*3*) were derived from a single coding sequence, presumably that of rat ErbB3. However, the extreme 5′-end of the rat *ErbB3* message was not represented in these isolated cDNAs. The remaining 5′-end of the rat *ErbB3* cDNA was therefore generated by a RT-PCR method (see Fig. 1).

(b) Deduced aa sequence of rat ErbB3

The 5′-cDNA generated by RT-PCR and three cDNA fragments isolated from the cDNA library were together subcloned to yield a full-length rat *ErbB3* cDNA. The deduced aa sequence of rat ErbB3 and the sequence deviations between human and rat ErbB3 are shown in Fig. 1. Overall there was a 90% aa identity between the human and rat sequences. Two short gaps in the sequence alignment indicated the deletion in the rat sequence of two aa in the presumed TM domain and one aa in the C-terminal autophosphorylation domain. The sequence encoding the putative PTK domain of the rat protein showed a 96% identity with that of the human protein. The rat *ErbB3* gene product is predicted to consist of 1339 aa (147 578 Da).

Comparison of the deduced rat ErbB3 aa sequence with the consensus sequence of the catalytic domains of known PTK (Hanks and Quinn, 1991) showed two substitutions of invariantly conserved aa (see Fig. 1). The residues Cys^{738} and His^{757} of the rat protein, which correspond respectively to Ala^{719} and Glu^{738} of the human epidermal growth factor (EGF) receptor, deviate from the canonical sequence. Notably, Asp^{832} of the rat ErbB3 sequence, which corresponds to Asp^{813} in the EGF receptor sequence, does agree with the consensus sequence, although it is found substituted in the human ErbB3 sequence by Asn^{834}. Given that the mutation of this conserved aspartate residue has been shown to abolish the PTK activity of the EGF receptor (Coker et al., 1994), the lack of this substitution in the rat protein could conceivably alter the catalytic properties of rat ErbB3 relative to those of the human protein. Recently, Prigent and Gullick (1994) employed site-directed mutagenesis to explore the consequences of the substitution in human ErbB3 of His^{759} and Asn^{834} for the normally conserved Glu and Asp residues. The low levels of PTK activity exhibited by wild-type ErbB3 in immune-complex kinase assays were not found to be enhanced by a $His^{759} \rightarrow Glu$ and $Asn^{834} \rightarrow Asp$ double aa substitution.

Other notable structural features are found in the C-terminal domain of rat ErbB3. Of particular interest is the presence of several candidate Tyr residue phosphorylation sites in specific sequence motifs that constitute the recognition sites for SH2 domain (second domain of Src homology) proteins (Cohen et al., 1995; Pawson, 1995). The SH2 domains found in various signal-transducing proteins (STP), such as PI 3-kinase, phospholipase C-γ1, GRB2 and SHC, have been shown to bind to activated growth factor receptors and related proteins that contain phosphorylated Tyr residues. It has become apparent that each distinct SH2 domain protein recognizes phospho-Tyr residues in a specific sequence context (Songyang et al., 1993). The sequence motif Tyr-Xaa-Xaa-Met, the consensus binding site sequence for PI 3-kinase, is found repeated seven times in the C-terminal domain of human ErbB3, and the seven repeats of this sequence are found intact in the C terminus of the deduced rat ErbB3 aa sequence (see Table 1). Two consensus binding sites for the GRB2 protein, Tyr-Met-Asn, are also found in the C-termini of both rat and human ErbB3. Also of interest is the overlapping of PI 3-kinase binding sites with these two GRB2 binding sites in the sequence element Tyr-Glu-Tyr-Met-Asn. Conceivably, these dual-specificity binding sites, depending upon which of the two Tyr residues were phosphorylated, could bind either PI 3-kinase or the GRB2 protein. A potential SH2 domain binding site for Src family PTK, Tyr-Glu-Glu-Met, is also present in both rat and human ErbB3 sequences. Another motif observed in both the rat and human sequences is Tyr-Val-Met-Pro, which if phosphorylated could constitute a binding site for the SH2 domain-containing protein tyrosine phosphatase SH-PTP2. Finally, the binding site for SHC previously identified in human ErbB3 (Prigent and Gullick, 1994) is again found in the rat sequence. The observed cross-species conservation of the aa sequences of these various SH2 domain binding sites is consistent with the assumption that ErbB3 interacts functionally with multiple STP.

Recently it has been determined that the SH3 domains found in various STP bind specifically to certain Pro-rich sequences (Kapeller et al., 1994; Ren et al., 1993). Two such sequences, Pro-Arg-Pro-Pro-Arg-Pro and Pro-Lys-Pro-Pro-Lys-Pro, found in the p85 subunit of PI 3-kinase are part of the identified binding sites for the SH3 domains of Src family PTK (Kapeller et al., 1994). Also, the sequence Pro-Xaa-Xaa-Pro-Pro-Pro-Xaa-Xaa-Pro as found twice in the 3BP-1 protein has been considered a consensus SH3 domain binding site (Ren et al., 1993). The first element, Pro-Arg-Pro-Pro-Arg-Pro, is found within the C terminus of rat ErbB3, with the corresponding sequence in the human protein being Pro-His-Pro-Pro-Arg-Pro (see Table 1). A sequence very similar to the second element, Pro-Leu-His-Pro-Val-Pro-Ile-Met-Pro, is found in the human ErbB3 C terminus, although the corresponding rat sequence, Pro-Leu-His-Pro-Met-Ala-Ile-Val-Pro, is less similar. Presumably these proline-rich sequences of rat ErbB3 could interact directly with SH3 domain proteins, and perhaps in cooperation with the identified SH2 domain binding motifs could mediate high-affinity interactions with proteins

TABLE I

Potential binding sites for SH2 and SH3 domain proteins identified in human and rat ErbB3 aa sequences

SH2/SH3 domain protein[a]	Binding site[b]	Human ErbB3[c]	Rat ErbB3
p85 (PI 3-kinase) (SH2)	Y(M/X)XM	Y^{941}MVM	Y^{939}MVM
		Y^{1054}MPM	Y^{1051}MPM
		Y^{1197}EYMN	Y^{1194}EYMN
		Y^{1222}EYM	Y^{1219}EYM
		Y^{1260}EYMN	Y^{1257}EYMN
		Y^{1276}AAM	Y^{1273}AAM
		Y^{1289}EEM	Y^{1286}EEM
GRB2/Sem-5 (SH2)	YMN	YEY^{1199}MN	YEY^{1196}MN
		YEY^{1262}MN	YEY^{1259}MN
SHC (SH2)	NPXY[d]	NPDY^{1328e}	NPDY^{1325}
SH-PTP2 (SH2)	YVXP	Y^{1159}VMP	Y^{1156}VMP
Src family kinases (SH2)	YEEM	Y^{1289}EEM	Y^{1286}EEM
p85 (SH3)	P(R/K)PP(R/K)P[f]	P^{1206}HPPRP	P^{1209}RPPRP

[a] Specific proteins containing SH2 or SH3 domains (second or third domain of Src homology, respectively) as indicated in parentheses.
[b] Consensus SH2 domain binding motifs selected from random peptide libraries with specific SH2 domain probes (Songyang et al., 1993). Phosphorylated Tyr residues (Y) in binding site sequences are indicated in bold.
[c] Sites identified in the published aa sequence of human ErbB3 (Kraus et al., 1989; Plowman et al., 1990) with numbering according to Kraus et al. (1989).
[d] Consensus SHC binding site as previously described (Prigent and Gullick, 1994).
[e] Identified SHC binding site in human ErbB3 (Prigent and Gullick, 1994).
[f] From two identified Pro-rich SH3 domain binding site sequences in the p85 subunit of PI 3-kinase (Kapeller et al., 1994).

containing both SH2 and SH3 domains, such as GRB2, p85 and Src family PTK.

(c) Cellular expression of the isolated *ErbB3* cDNA

In order to verify that the full-length cDNA generated by ligation of the several isolated cDNA molecules encoded a protein consistent with the predicted structure of ErbB3, this cDNA was subcloned into a mammalian cell expression vector and transfected into cultured mouse NIH-3T3 fibroblasts. Lysates of the transiently transfected fibroblasts were analyzed by SDS–PAGE and immunoblotting with an ErbB3-specific Ab. A relatively high level of approx. 180-kDa immunoreactive protein was detected in lysates of fibroblasts transfected with the rat *ErbB3* expression vector under conditions of optimal pH (see Fig. 2). Although the molecular mass of recombinant ErbB3 (re-ErbB3) indicated by SDS–PAGE was significantly greater than that predicted by the cDNA sequence, it was similar to that of endogenous ErbB3 of human breast cancer cells (data not shown). Apparently both the rat and human ErbB3 proteins are subject to glycosylation.

(d) Activation of rat re-ErbB3 by the HRG-β peptide

The EGF-related polypeptide HRG-β1, previously characterized as an activating ligand for human ErbB3 (Carraway et al., 1994; Sliwkowski et al., 1994), was tested as an activator of rat re-ErbB3 in transfected NIH-3T3 fibroblasts. While a constitutive phosphorylation of re-ErbB3 was detected by immunoprecipitation with an

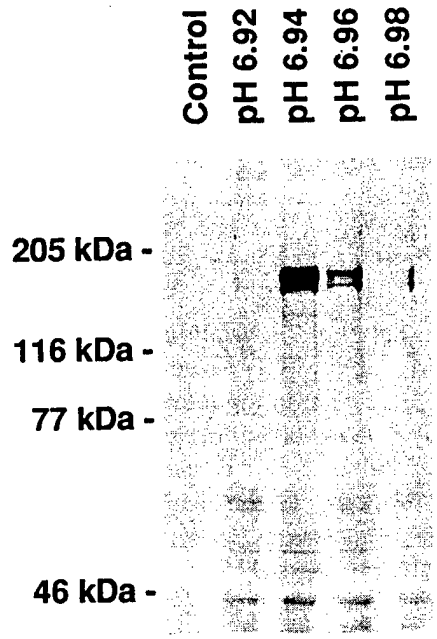

Fig. 2. Transient expression of the rat *ErbB3* cDNA in mouse NIH-3T3 fibroblasts. Subconfluent NIH-3T3 cells in 75-cm^2 flasks were transfected with 20 μg of pcDNA3-*ErbB3* plasmid per flask by a modified Ca·phosphate method (Sambrook et al., 1989). Buffers of slightly varying pH were used to optimize transfection efficiency. At 48 h post-transfection cells were detergent-lysed and samples of the lysates subjected to 0.1% SDS-7% PAGE and immunoblotting with ErbB3-specific monoclonal Ab 2F12 (Kim et al., 1994) (NeoMarkers, Fremont, CA, USA). Immunoblots were visualized with the ECL detection system (Amersham, Arlington Heights, IL, USA). Parental NIH-3T3 cells were analyzed for comparison (Control).

ErbB3-specific Ab and immunoblotting with antiphospho-Tyr, challenge of the transfected fibroblasts with HRG-β1 clearly enhanced the phosphorylation of re-ErbB3 (see Fig. 3). Quantitation of immunoblots by densitometry yielded the average fold-stimulation of re-ErbB3 phosphorylation as 3.3 ± 0.9 ($n=4$). Considering that previous studies have demonstrated that ErbB2 and ErbB3 function as coreceptors for the HRG polypeptide (Sliwkowski et al., 1994) and that ErbB4 is also responsive to HRG (Plowman et al., 1993b), it is possible that an endogenous ErbB family member present at low levels in the transfected NIH-3T3 fibroblasts was involved in the observed stimulation of ErbB3 phosphorylation by HRG. As we have in numerous attempts failed to detect intrinsic PTK activity in the rat *ErbB3* gene product (data not shown), we assume that a distinct PTK(s) was responsible for both the constitutive and HRG-stimulated phosphorylation of re-ErbB3 in the transfected fibroblasts. Notably, we and others have demonstrated an EGF-dependent phosphorylation of ErbB3 in cultured cells containing both the EGF receptor and ErbB3 (Kim et al., 1994; Soltoff et al., 1994).

As previously documented by studies of human ErbB3 (Fedi et al., 1994; Kim et al., 1994; Soltoff et al., 1994), the p85 subunit of PI 3-kinase was found to immunoprecipitate with phosphorylated rat re-ErbB3 (see Fig. 3). Exposure of the transfected fibroblasts to HRG-β1 significantly increased the quantity of p85 detected in ErbB3 immunoprecipitates. The HRG-dependent binding of this STP protein was possibly mediated by the phosphorylation of one or more of the Tyr-Xaa-Xaa-Met p85-binding motifs present in the ErbB3 C terminus.

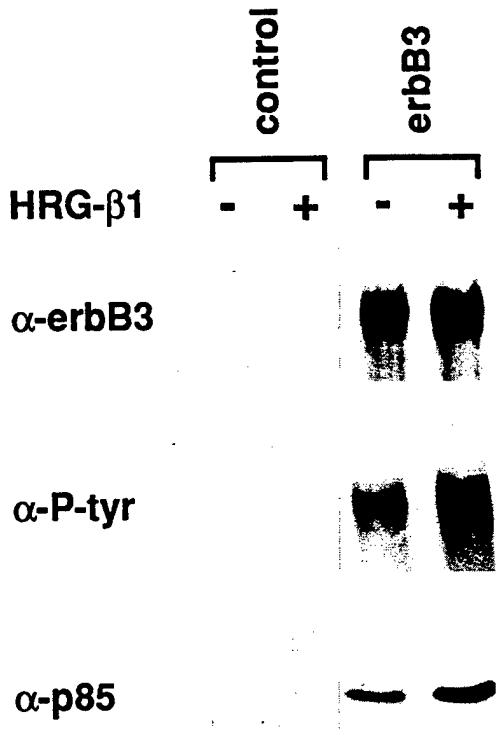

Fig. 3. Activation of rat re-ErbB3 protein by HRG-β1. Mouse NIH-3T3 fibroblasts were transfected with the pcDNA3-*ErbB3* expression vector (see Fig. 2) and cultured under neomycin (G418) selection. Cells stably expressing the rat *ErbB3* cDNA were serum-starved for 16 h and then incubated for 5 min at 37°C in the presence or absence of an HRG-β1 re-peptide fragment (H.-H. K., J.G.K., and S. Campion, data not shown) incorporating aa 177 to 241 of the HRG-β1 precursor molecule (Holmes et al., 1992) at a concentration of 50 nM. Control cells transfected with the parent pcDNA3 expression vector (Invitrogen, San Diego, CA, USA) were similarly treated. Cell lysates were immunoprecipitated with ErbB3-specific monoclonal Ab 2F12, and immunoprecipitates were subjected to 0.1% SDS-7%-PAGE and immunoblotting with either Ab 2F12 (α-ErbB3), antiphospho-Tyr Ab PY20 (α-P-tyr) (Leinco Technologies, St. Louis, MO, USA), or a polyclonal Ab recognizing the p85 subunit of PI 3-kinase (α-p85) (Transduction Laboratories, Lexington, KY, USA) as indicated.

(e) Conclusions

(*1*) A rat liver cDNA encoding a 1339-aa protein with 90% sequence identity with human ErbB3 has been isolated. Presumably derived from the rat *ErbB3* gene, this cDNA was expressed in cultured mouse NIH-3T3 fibroblasts, in which a high level of the approx. 180-kDa rat re-ErbB3 protein could be generated.

(*2*) The ErbB3 protein isolated by immunoprecipitation of cell lysates was constitutively phosphorylated on Tyr residues. Prior challenge of the cells with HRG-β1 led to an enhanced ErbB3 phosphorylation, which indicated that rat ErbB3 may be a functional HRG receptor.

(*3*) Immunoprecipitation experiments indicated that a fraction of the total p85 protein in the transfected fibroblasts was constitutively associated with rat ErbB3. Challenge of the transfected cells with HRG-β1 augmented the quantity of ErbB3-associated p85. Together these results indicated that the isolated rat *ErbB3* cDNA encodes a functional receptor protein that is responsive to HRG and can transduce signals to the PI 3-kinase pathway.

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Mutation of a Shc Binding Site Tyrosine Residue in ErbB3/HER3 Blocks Heregulin-dependent Activation of Mitogen-activated Protein Kinase*

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Ulka Vijapurkar, Kunrong Cheng‡, and John G. Koland§

From the Department of Pharmacology, the University of Iowa College of Medicine, Iowa City, Iowa 52242-1109

The ErbB2 and ErbB3 proteins together constitute a functional coreceptor for heregulin (neuregulin). Heregulin stimulates the phosphorylation of both coreceptor constituents and initiates a variety of other signaling events, which include phosphorylation of the Shc protein. The role of Shc in heregulin-stimulated signal transduction through the ErbB2·ErbB3 coreceptor was investigated here. Heregulin was found to promote ErbB3/Shc association in NIH-3T3 cells expressing endogenous ErbB2 and recombinant ErbB3. A mutant ErbB3 protein was generated in which Tyr-1325 in a consensus Shc phosphotyrosine-binding domain recognition site was mutated to Phe (ErbB3-Y/F). This mutation abolished the association of Shc with ErbB3 and blocked the activation of mitogen-activated protein kinase by heregulin. Whereas heregulin induced mitogenesis in NIH-3T3 cells transfected with wild-type ErbB3 cDNA, this mitogenic response was markedly attenuated in NIH-3T3 cells transfected with the ErbB3-Y/F cDNA. These results showed a specific interaction of Shc with the ErbB3 receptor protein and demonstrated the importance of this interaction in the activation of mitogenic responses by the ErbB2·ErbB3 heregulin coreceptor complex.

The ErbB3/HER3 receptor protein is a member of the ErbB/HER family of growth factor receptors (1), the prototype of which is the epidermal growth factor (EGF)[1] receptor (ErbB1/HER1). Like other members of this family, the ErbB3 protein consists of an extracellular ligand binding domain, a transmembrane domain, an intracellular protein tyrosine kinase domain, and a C-terminal phosphorylation domain. Human heregulins (2) or their rat counterparts, the Neu differentiation factors (3), have been identified as a family of ligands for this receptor. ErbB3 is unique among ErbB/HER family members in that it has an impaired protein tyrosine kinase activity, which has been attributed to the substitution of amino acid residues invariantly conserved in protein tyrosine kinases (4, 5). However, ErbB3 tyrosine residue phosphorylation is observed when ErbB3 is coexpressed with other ErbB family members, apparently through the formation of heterodimeric receptor complexes (6, 7). Cells coexpressing EGF receptor and ErbB3 show an EGF-dependent ErbB3 phosphorylation (8, 9). Heregulin-stimulated phosphorylation of both ErbB2 and ErbB3 occurs in cells coexpressing these proteins (10–12), and although ErbB2 itself does not bind heregulin, ErbB2 and ErbB3 cooperate in the formation of a high affinity heregulin coreceptor complex (10). In addition, heregulin-dependent phosphorylation of EGF receptor and ErbB2 has been attributed to cross-phosphorylation by the kinase-intact heregulin receptor ErbB4 (13, 14).

Among the heterodimers formed within the ErbB family, the ErbB2·ErbB3 coreceptor complex is believed to elicit the most potent mitogenic signal (7, 11, 15). The contribution of ErbB3 to the mitogenic potential of ErbB family coreceptors might be enhanced by its unique C-terminal phosphorylation domain, which possesses several consensus sequences for the binding of signal-transducing proteins, including phosphoinositide (PI) 3-kinase, Grb2, Shc, SH-PTP2, and Src family protein tyrosine kinases (16). Notably, this domain contains six repeats of the consensus motif, Tyr-Xaa-Xaa-Met (YXXM), for binding to the p85 subunit of PI 3-kinase (17, 18). The role of PI 3-kinase in signal transduction by ErbB family coreceptors has begun to be clarified. The EGF-dependent association of PI 3-kinase with the ErbB3 protein has been observed in cancer cells expressing high levels of both EGF receptor and ErbB3 (8, 9). Also, a heregulin-dependent association of PI 3-kinase with ErbB3 has been seen in the context of the ErbB2·ErbB3 coreceptor, and the resulting activation of PI 3-kinase has been shown to be important for heregulin-stimulated mitogenesis (11).

Like other ErbB family members, the ErbB3 protein incorporates a consensus motif, Asn-Pro-Xaa-Tyr (NPXY), for binding to the Shc protein. Shc is an adapter protein that contains a C-terminal SH2 domain and an N-terminal phosphotyrosine-binding domain. The phosphotyrosine-binding domain of Shc specifically binds to phosphotyrosine in the NPXY sequence context (19–22) and mediates the binding of Shc to the EGF (23–25) and insulin (25, 26) receptors. Receptor-associated Shc is rapidly phosphorylated (27–29) and subsequently binds a Grb2·Sos complex, which results in the translocation of the complex to the plasma membrane (30, 31). Sos, a guanine nucleotide exchange protein, then activates Ras (32, 33), which in turn stimulates the mitogen-activated protein kinase (MAPK) cascade (34, 35). Shc has been implicated in mitogenic signaling by epidermal growth factor (36), platelet-derived

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‡ Present address: Dept. of Cell Biology, Neurobiology and Anatomy, Loyola University Medical Center, Maywood, IL 60153.

§ To whom correspondence should be addressed. Tel.: 319-335-6508; Fax: 319-335-8930.

[1] The abbreviations used are: EGF, epidermal growth factor; Erk, extracellularly regulated kinase; DMEM, Dulbecco's modified Eagle's medium; Grb2, growth factor receptor-bound protein 2; GST, glutathione *S*-transferase; GST-B3, GST fusion protein incorporating rat ErbB3 residues 1311–1339; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NPXY, Asn-Pro-Xaa-Tyr sequence motif; PAGE, polyacrylamide gel electrophoresis; PI, phosphoinositide; WT, wild-type; Y/F, Tyr → Phe amino acid substitution; YXXM, Tyr-Xaa-Xaa-Met sequence motif.

growth factor (37), nerve growth factor (38), and insulin (39) receptors.

The Shc protein has been shown to associate with phosphorylated ErbB3 (28, 40), and heregulin has been found to stimulate the phosphorylation of Shc (40). These findings suggest a possible contribution of the Shc signaling pathway to heregulin-stimulated mitogenesis. Synthetic phosphopeptide competition experiments have indicated that Tyr-1309 in human ErbB3 is the binding site of Shc (28). By mutating the corresponding tyrosine residue in the putative Shc binding site of the rat ErbB3 receptor protein, we have in the present study examined the heregulin-stimulated interaction of Shc with the ErbB3 receptor, and we have investigated the role of Shc in mitogenesis mediated by the ErbB2·ErbB3 coreceptor complex.

EXPERIMENTAL PROCEDURES

Materials—NIH-3T3 cells were purchased from American Type Culture Collection. LipofectAMINE transfection reagent was obtained from Life Technologies, Inc. Recombinant heregulin-β1 and antibodies recognizing ErbB2 (Ab-1) and ErbB3 (2C3, 2F12) were purchased from NeoMarkers. Anti-phosphotyrosine (PY20), recombinant PY20 conjugated to horseradish peroxidase, anti-Shc, and anti-Grb2 were purchased from Transduction Laboratories. Anti-p85 was purchased from Upstate Biotechnology. A mitogen-activated protein kinase-specific antibody recognizing both Erk1 and Erk2 isoforms (Zymed Laboratories Inc.) and distinct Erk1-specific and Erk2-specific antibodies (Santa Cruz) were also procured. Recombinant platelet-derived growth factor-BB and wortmannin were purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech. [γ-^{32}P]ATP (3000 Ci/mmol) and [*methyl*-^{3}H]thymidine (90 Ci/mmol) were acquired from NEN Life Science Products. The recombinant EGF receptor protein tyrosine domain, consisting of amino acid residues 645–972 of the parent receptor, was expressed with the baculovirus/insect system and purified as described previously (5).

Generation of an ErbB3 Tyr-1325 → Phe Mutant Protein—The rat ErbB3 cDNA (16) was mutated by use of the Ex-Site Mutagenesis kit from Stratagene. A tyrosine codon corresponding to amino acid 1325 was replaced with a phenylalanine codon with a 33-base pair reverse mutagenic primer 5′-GGGAAAAGCCGGCTGTGCCAGAAATCGGGGTTG-3′ and the ErbB3 expression plasmid pcDNA3-B3 (16) as the template for the polymerase chain reaction mutagenesis. The altered region of the cDNA was subcloned into the parent expression vector to yield the mutant ErbB3 receptor cDNA expression vector (pcDNA3-B3-Y/F). The affected region was sequenced to verify the accuracy of polymerase chain reaction amplification.

Cell Culture—NIH-3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. After transfection with the pcDNA3-B3-Y/F mutant expression plasmid using LipofectAMINE reagent, stable NIH-3T3 clones were selected with Geneticin (G418) and screened for the expression of the mutant receptor protein by Western blotting. A stable NIH-3T3 cell line expressing ErbB3-WT was isolated as described previously (16). For some [^{3}H]thymidine incorporation assays nonclonal pools of NIH-3T3 cells transfected with pcDNA3-B3-WT and pcDNA3-B3-Y/F were grown under Geneticin selection. Equivalent expression of wild-type and mutant receptors was verified by immunoblotting.

Cell Stimulation, Immunoprecipitation, and Immunoblotting—Prior to stimulation with growth factor, cells were starved for 18 h in low serum medium (DMEM containing 0.1% fetal bovine serum). Starved cells were washed once with low serum medium and incubated with heregulin-β1 (1 nM final concentration) diluted in culture medium containing 0.1% bovine serum albumin, or the dilution vehicle, for 5–7 min at 37 °C. Cells were washed immediately with ice-cold phosphate-buffered saline and lysed with Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Hepes/Na, 150 mM sodium chloride, 2 mM EDTA, 3 mM EGTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, pH 7.4). The whole cell lysates were centrifuged for 10 min at 13,000 × *g*. After protein concentration was assayed, the supernatants were immunoprecipitated with appropriate antibodies (8). The immunoprecipitates and cell lysate samples were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and detected with the indicated antibodies by ECL luminography.

In Vitro Binding Assays—A C-terminal peptide fragment of ErbB3 (residues 1311–1339) containing Tyr-1325 was generated as a GST fusion protein (GST-B3) and purified as described previously (41). GST-B3 or GST (65 pmol each) was incubated in buffer A (20 mM Hepes/Na, 50 mM sodium chloride, 10% (v/v) glycerol, pH 7.4) supplemented with 10 mM $MnCl_2$, 0.1% Triton X-100, and 0.1 μg of EGF receptor protein tyrosine kinase domain (5) in the absence or presence of 50 μM ATP for 30 min at 22 °C (total volume 10 μl). The mixtures were diluted into 375 μl of lysate from NIH-3T3 cells (2 mg/ml protein), incubated for 30 min on ice, and then allowed to bind glutathione-agarose (100 μl of a 1:1 suspension in buffer A) for 1 h at 4 °C. The agarose suspensions were centrifuged for 1 min at 600 × *g*. The pellets were washed twice in 500 μl of ice-cold Nonidet P-40 lysis buffer and then suspended in gel sample buffer. Pellets and cell lysate samples (20 μg of protein) were resolved by SDS-PAGE and immunoblotted with anti-Shc and anti-phosphotyrosine.

Mitogen-activated Protein Kinase Assay—Mitogen-activated protein kinase (MAPK) from cells stimulated with heregulin or control vehicle was immunoprecipitated with a combination of Erk1 and Erk2 antibodies as described above. The washed immunoprecipitates were suspended in 30 μl of reaction buffer containing 10 mM Hepes/ Na, 10 mM $MgCl_2$, pH 7.4, and 8 μg of myelin basic protein (MBP). The reaction was initiated by adding 3 μl of 100 μM ATP containing 5 μCi of [γ-^{32}P]ATP and incubated for 15 min at 30 °C. The reaction was quenched with sample buffer, and the proteins were subjected to SDS-PAGE. The gel was subsequently dried, exposed to autoradiographic film, and MBP phosphorylation quantified by scintillation counting of excised gel bands. In the MAPK gel shift assay, cell lysate supernatants from heregulin-, platelet-derived growth factor-, or vehicle-stimulated cells were subjected to Western blotting with an antibody recognizing both the Erk1 and Erk2 isoforms of MAPK. Here in SDS-PAGE the amount of bisacrylamide in the gel was reduced (acrylamide:bisacrylamide, 30:0.04). and the electrode buffer was twice-concentrated (42).

[^{3}H]Thymidine Incorporation Assay—Cells were plated at a density of 5 × 10^{5}/well in 6-well dishes, grown for 24 h, and then serum-deprived for 18 h in DMEM containing 0.1% fetal bovine serum. Cells were then stimulated with varying concentrations of heregulin-β1 for 18 h, after which 0.5 μCi/ml of [*methyl*-^{3}H]thymidine was added to each well, and the cells were further incubated for 4 h. For experiments with wortmannin, either Me_2SO or wortmannin (100 nM) in Me_2SO was added 30 min prior to stimulation of cells with either vehicle or heregulin (10 nM). Cells were then washed twice with cold phosphate-buffered saline, extracted with 5% trichloroacetic acid, and then solubilized in 0.1 M sodium hydroxide. The radioactivity incorporated into DNA was measured by scintillation counting.

RESULTS

Heregulin-dependent Phosphorylation of Wild-type and Mutant ErbB3 Proteins in Stably Transfected NIH-3T3 Cells—By using site-directed mutagenesis, we created a mutant ErbB3 protein in which the candidate Shc binding site residue, Tyr-1325 (28), was substituted with phenylalanine (ErbB3-Y/F). NIH-3T3 fibroblast cell lines that stably expressed high levels of the wild-type (ErbB3-WT) and mutant (ErbB3-Y/F) receptor proteins were isolated. To confirm the expression of the receptor proteins, cell lysates were analyzed by immunoprecipitation followed by Western blotting with an ErbB3-specific antibody. The transfected fibroblasts expressed comparable levels of wild-type and mutant ErbB3 proteins (Fig. 1*A*). Cells transfected with the parent expression vector did not express detectable ErbB3.

As we have previously observed (16), ErbB3-WT showed a constitutive phosphorylation on tyrosine residues that was enhanced by stimulation with heregulin-β1 (Fig. 1*A*). Phosphorylation of the ErbB3-Y/F mutant protein was enhanced to a similar extent as the wild-type protein. Treatment of the mock-transfected cells with heregulin induced no phosphorylation response. The heregulin-dependent phosphorylation of tyrosine residues in ErbB3 was presumably mediated by the ErbB2 protein tyrosine kinase, endogenously present in the NIH-3T3 fibroblasts (Fig. 1*B*). The endogenous ErbB2 protein in NIH-3T3 cells expressing either ErbB3-WT or ErbB3-Y/F was phos-

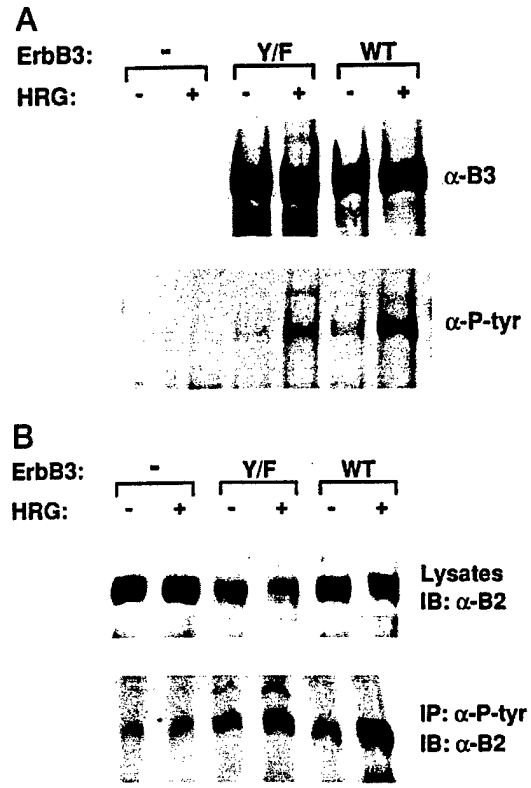

FIG. 1. **Expression and heregulin-mediated phosphorylation of ErbB2 and ErbB3 in stably transfected NIH-3T3 cell lines.** NIH-3T3 cells were transfected with either the parent pcDNA3 expression vector (−) or pcDNA3 incorporating wild-type (*WT*) and Tyr-1325 → Phe mutant (Y/F) ErbB3 cDNAs. Mock-transfected cells and cells expressing ErbB3-Y/F and ErbB3-WT were serum-starved and stimulated with vehicle or 1 nM heregulin-β1. *A*, cell lysates containing 1 mg of protein were immunoprecipitated with ErbB3-specific antibody, and the immunoprecipitates were subjected to SDS-PAGE and immunoblotting with either ErbB3-specific antibody (*α-B3*) or anti-phosphotyrosine antibody (*α-P-tyr*). *B*, alternatively, cell lysates were blotted with ErbB2-specific antibody (*α-B2*) or immunoprecipitated (*IP*) with a phosphotyrosine-specific antibody (*α-P-Tyr*) and then immunoblotted (*IB*) with ErbB2-specific antibody.

phorylated on tyrosine residues, and this phosphorylation was augmented in response to heregulin (Fig. 1*B*), which was consistent with the observation that ErbB2 and ErbB3 function as heregulin coreceptors (10, 43).

The Tyr-1325 → Phe Point Mutation in ErbB3 Abolishes Heregulin-dependent ErbB3/Shc Association—To assay the association of Shc with the wild-type and mutant ErbB3 receptor proteins, lysates of stably transfected NIH-3T3 cells were immunoprecipitated with an Shc-specific antibody and subsequently immunoblotted with an ErbB3-specific antibody. Lysates from mock-transfected cells and cells expressing either the mutant or wild-type receptor protein showed the presence of each isoform of Shc, p46, p52, and p66, in similar amounts across the cell lines (Fig. 2*A*). From cells expressing the wild-type receptor, the ErbB3 protein coimmunoprecipitated with Shc, which suggested that Shc constitutively associated with ErbB3. However, this ErbB3/Shc association was significantly enhanced following stimulation with heregulin. In contrast, Shc immunoprecipitates from cells expressing ErbB3-Y/F showed no presence of the ErbB3-Y/F protein (Fig. 2*B*). Thus, the mutation of a single tyrosine in the NPXY sequence motif in the ErbB3 receptor abolished association of Shc with ErbB3. Mock-transfected cells showed no heregulin-dependent ErbB3/Shc association. Interestingly, no association of Shc with ErbB2 was evident in any of the cells (Fig. 2*B*) (see "Discussion").

Heregulin-stimulated Shc Phosphorylation and Shc/Grb2 Association Is Significantly Attenuated in NIH-3T3 Cells Expressing ErbB3-Y/F—Since the Tyr → Phe mutation blocked the interaction of Shc with the ErbB3 receptor, we examined the effect of this mutation on heregulin signaling by the ErbB2·ErbB3 coreceptor. To investigate potential heregulin-stimulated Shc phosphorylation, Shc immunoprecipitates were probed with a phosphotyrosine-specific antibody. An increase in the phosphorylation of the Shc proteins was seen in response to heregulin in cells expressing the wild-type receptor. Among the three isoforms of Shc, p52 seemed to be preferentially phosphorylated in response to heregulin. In cells expressing ErbB3-Y/F the heregulin-induced phosphorylation of Shc was significantly reduced as compared with cells expressing the wild-type receptor (Fig. 2*C*). Heregulin did not stimulate Shc phosphorylation in the mock-transfected cells.

Anti-Shc immunoprecipitates were also probed with a Grb2-specific antibody. Grb2 was found to be constitutively associated with Shc, but this association was increased in response to heregulin in cells expressing ErbB3-WT. No increase in heregulin-stimulated Shc/Grb2 association was seen in cells expressing the ErbB2-Y/F mutant receptor. Vector-transfected cells also showed no heregulin-dependent Shc/Grb2 association (Fig. 2*C*). These results indicated that Shc phosphorylation and Shc/Grb2 association were potentiated by the binding of Shc to ErbB3, which was apparently mediated by Tyr-1325 in the ErbB3 C terminus.

A Phosphorylated ErbB3 C-terminal Peptide Interacts with Shc Proteins in Vitro—To determine whether Tyr-1325 in the ErbB3 C terminus could when phosphorylated serve as a binding site for the Shc protein, we expressed a short C-terminal peptide fragment of ErbB3 (residues 1311–1339) containing only one tyrosine residue, Tyr-1325, as a GST fusion protein (GST-B3), and we used this protein in *in vitro* binding assays. Here the GST-B3 fusion protein was first phosphorylated with a recombinant EGF receptor protein tyrosine kinase domain (5) and then incubated with lysates of NIH-3T3 cells containing the Shc proteins. After precipitation of the phosphorylated GST-B3 protein with glutathione-agarose, associated Shc proteins were detected by Western blotting (Fig. 2*D*). Control experiments showed that the GST domain was not phosphorylated under these conditions and did not significantly interact with the Shc proteins. Also, the interaction of Shc with GST-B3 was dependent upon prior phosphorylation of the fusion protein. These results indicated that the C-terminal NPXY motif in ErbB3 could serve when phosphorylated as an Shc-binding site.

Heregulin-stimulated Activation of MAPK Is Impaired in NIH-3T3 Cells Expressing the ErbB3-Y/F Mutant Protein—Receptor-mediated Shc phosphorylation and Shc/Grb2 association would be predicted to result in the activation of the Ras/MAPK signaling pathway. Potential heregulin-stimulated activation of MAPK was characterized in NIH-3T3 cells expressing ErbB2·ErbB3 coreceptors (Fig. 3). The ErbB3-Y/F receptor protein, which failed to interact with Shc, was used to examine the involvement of Shc in the activation of MAPK via the ErbB2·ErbB3 coreceptor. The activation of MAPK in the NIH-3T3 transfectants was detected by gel mobility shift assays (Fig. 3*A*) and *in vitro* phosphorylation assays employing the exogenous substrate myelin basic protein (MBP) (Fig. 3*B*). In NIH-3T3 cells expressing ErbB3-WT, MAPK was clearly activated in response to heregulin. This was evident by the retarded migration of both the p42 (Erk2) and p44 (Erk1) isoforms of MAPK in gel shift assays (Fig. 3*A*). Also, MAPK immunoprecipitates from heregulin-stimulated cells expressing ErbB3-WT showed strong MBP phosphorylation in immune

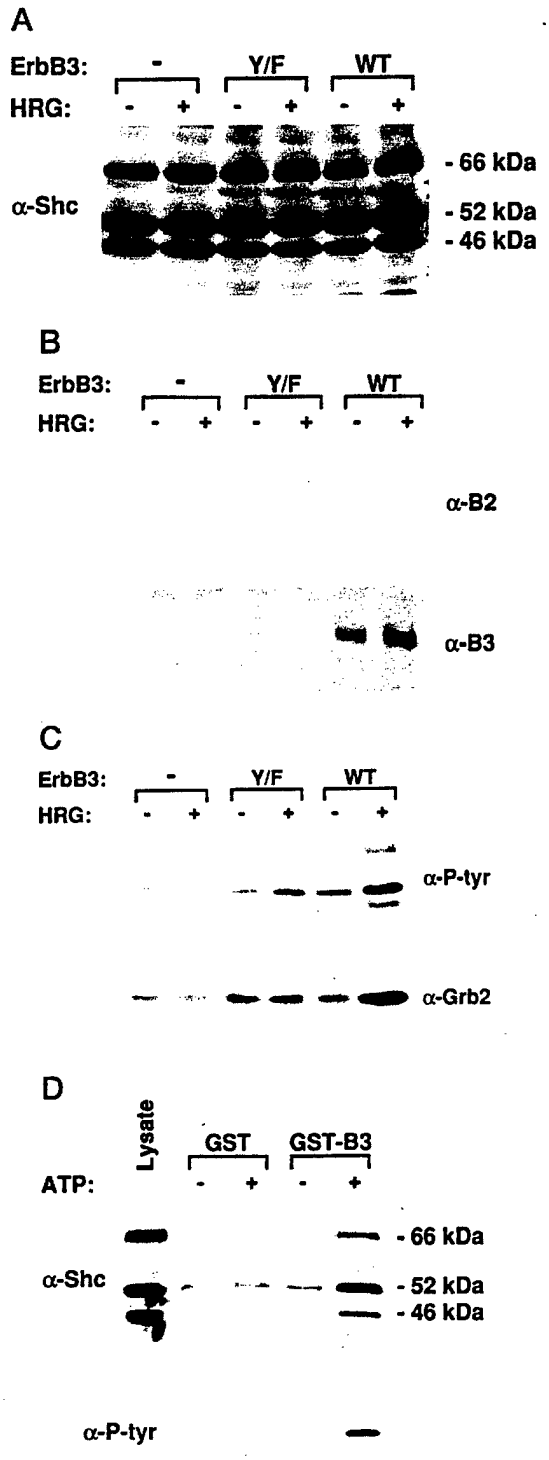

FIG. 2. **Heregulin-stimulated ErbB3/Shc association, Shc phosphorylation, and Shc/Grb2 association.** NIH-3T3 cells transfected with vector, ErbB3-WT, or ErbB3-Y/F cDNAs were treated as described in Fig. 1. *A*, lysates from cells stimulated with heregulin or control vehicle were probed with an Shc-specific antibody (*α-Shc*). All three isoforms of Shc (p46, p52, and p66) were evident. *B*, Shc was immunoprecipitated from the lysates with a Shc-specific antibody, and the immunoprecipitates were immunoblotted with either ErbB2-specific (*α-B2*) or ErbB3-specific (*α-B3*) antibody. *C*, Shc immunoprecipitates were also immunoblotted with an anti-phosphotyrosine-horseradish peroxidase conjugate (*α-P-tyr*) or a Grb2-specific antibody (*α-Grb2*). *D*, *in vitro* association of the phosphorylated ErbB3 C terminus with Shc proteins. GST-B3 and GST (65 pmol each) were incubated under phosphorylating (+ATP) or nonphosphorylating (−ATP) conditions, allowed to interact with Shc proteins from NIH-3T3 cell lysates, and then precipitated with glutathione-agarose. Association of Shc proteins with precipitated GST-B3 or GST was detected by immunoblotting with a Shc-specific antibody (*α-Shc*). An aliquot of the original cell lysates corresponding to ~1/40 of that in the binding assay was also analyzed and shown for comparison. Phosphorylation of the ErbB3 C-terminal peptide was detected by immunoblotting with anti-phosphotyrosine (*α-P-tyr*).

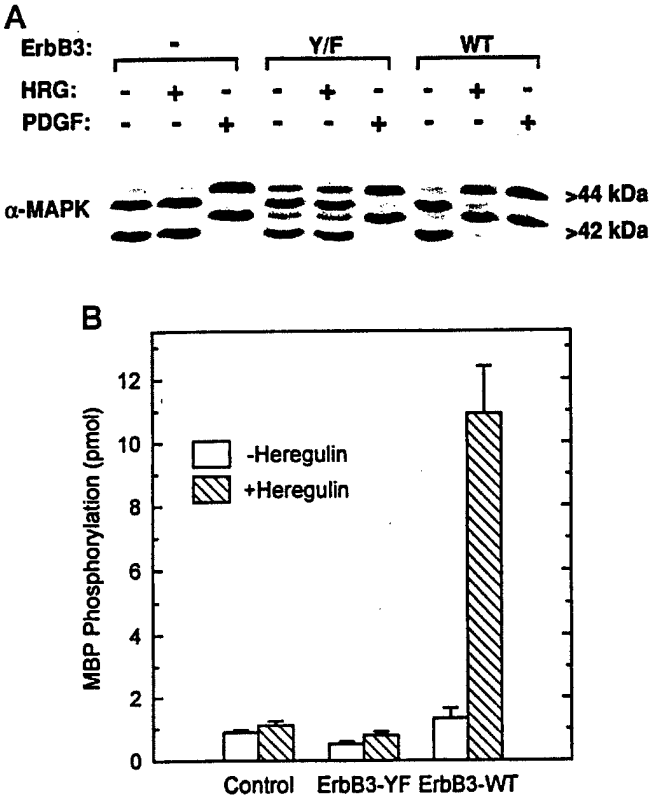

FIG. 3. **Heregulin-stimulated activation of mitogen-activated protein kinase.** *A*, cells stimulated with heregulin (1 nM), platelet-derived growth factor (50 ng/ml), or vehicle were lysed as described in Fig. 1. Cell lysates containing 70 μg of total protein were subjected to a gel mobility shift assay of MAPK activation as described under "Experimental Procedures." Both the p42 and p44 isoforms of MAPK are indicated. The appearance of more slowly migrating bands in cells transfected with wild-type ErbB3 cDNA in response to heregulin and in all three cell lines in response to platelet-derived growth factor indicated the activation of the MAPK isoforms. *B*, alternatively, MAPK was immunoprecipitated from heregulin-stimulated and control cells, and the immunoprecipitates were subjected to *in vitro* MAPK assays with MBP and [γ-^{32}P]ATP as substrates. *Error bars* represent the standard error of three independent experiments.

complex kinase assays (Fig. 3*B*). In contrast, NIH-3T3 cells expressing ErbB3-Y/F showed no MAPK activation in response to heregulin. Since NIH-3T3 fibroblasts endogenously express receptors for platelet-derived growth factor, it was of interest to see whether this factor stimulated the activation of MAPK in the various transfected cell lines. MAPK was clearly activated in response to platelet-derived growth factor in the mock-transfected cells and in cells expressing either ErbB3-Y/F or ErbB3-WT, as was evident by the retarded migration of both the p42 and p44 isoforms of MAPK in the gel shift assay (Fig. 3*A*). Possibly, the failure of the ErbB3-Y/F protein to activate MAPK in response to heregulin resulted from its inability to interact with Shc and mediate an Shc/Grb2 association.

Association of PI 3-Kinase with Wild-type and Mutant ErbB3 Proteins in Transfected NIH-3T3 Fibroblasts—Previous studies have reported an association of PI 3-kinase with the ErbB3 protein in both the EGF receptor·ErbB3 (8, 9) and ErbB2·ErbB3 (11) coreceptor contexts. Given that ErbB3-Y/F failed to both interact with Shc and activate MAPK, we sought to determine if the mutant receptor could still associate with PI

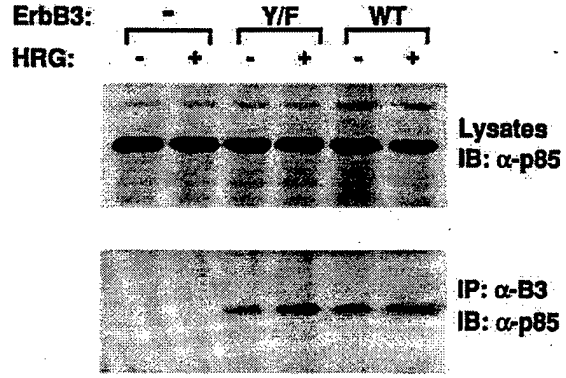

FIG. 4. **Association of PI 3-kinase with ErbB3-WT and ErbB3-Y/F in stably transfected NIH-3T3 fibroblasts.** Transfected cells were treated as described in Fig. 1. Lysates from vehicle- or heregulin-stimulated cells were probed with an antibody recognizing the p85 regulatory subunit of PI 3-kinase (α-p85). Cell lysates containing 1 mg of protein were also immunoprecipitated (*IP*) with ErbB3-specific antibody (α-B3), and the immunoprecipitates were subjected to SDS-PAGE and immunoblotting (*IB*) with p85-specific antibody.

3-kinase and therefore potentially signal through the PI 3-kinase pathway. Immunoblotting analyses of ErbB3 immunoprecipitates from control cells and cells expressing either ErbB3-WT or ErbB3-Y/F showed the presence of the p85 regulatory subunit of PI 3-kinase (Fig. 4), which indicated that the mutant ErbB3 protein retained its ability to interact with PI 3-kinase. Heregulin-stimulated association of p85 with ErbB3 was variable, which could have been due to the high basal association seen in the transfected NIH-3T3 cells.

Heregulin-stimulated DNA Synthesis in NIH-3T3 Cells Expressing ErbB3-WT and ErbB3-Y/F—In order to determine whether the wild-type and mutant ErbB3 proteins mediated a mitogenic response to heregulin, cellular DNA synthesis was analyzed with a [^{3}H]thymidine incorporation assay. The results of a representative experiment are shown in Fig. 5*A*. Mock-transfected NIH-3T3 cells showed no enhanced [^{3}H]thymidine uptake in response to heregulin. Cells expressing ErbB3-WT showed a dose-dependent uptake of [^{3}H]thymidine with a significant stimulation seen at a 0.1 nM concentration of heregulin. Cells expressing ErbB3-Y/F showed an attenuated mitogenic response relative to those expressing the wild-type receptor protein. Interestingly, the high basal activity seen in the cells expressing ErbB3-WT was absent in cells expressing ErbB3-Y/F. Heregulin-stimulated [^{3}H]thymidine incorporation, defined as the difference between basal incorporation and that stimulated by 10 nM heregulin, was compared between cells expressing either ErbB3-Y/F or ErbB3-WT (see Fig. 6). In five separate experiments, heregulin-stimulated DNA synthesis mediated by ErbB3-Y/F was found to range between 15 and 60% that mediated by ErbB3-WT. Heregulin-stimulated DNA synthesis was also studied with nonclonal pools of cells transfected with ErbB3-WT and ErbB3-Y/F cDNAs to ensure that the attenuated mitogenic response seen with clonal cells expressing ErbB3-Y/F was not an effect of clonal variation. Fig. 5*B* shows the results of a representative experiment with nonclonal cells expressing moderate levels of the wild-type and mutant receptor. Nonclonal cells expressing ErbB3-Y/F showed a significantly attenuated mitogenic response when compared with cells expressing ErbB3-WT. These results indicated that the association of Shc with the ErbB3 protein and the ensuing activation of the Ras/MAPK signaling pathway contributed to the mitogenic potential of the ErbB2·ErbB3 heregulin coreceptor.

Effect of Wortmannin on Heregulin-stimulated [^{3}H]Thymidine Incorporation—Given that the ErbB3-Y/F protein retained the ability to associate with the p85 regulatory subunit of PI 3-kinase (Fig. 4), it was considered that activation of the PI 3-kinase pathway might account for the residual mitogenic activity seen in cells expressing ErbB3-Y/F. To determine the contribution of PI 3-kinase to the stimulation of DNA synthesis by heregulin, we examined the effect of wortmannin, a PI 3-kinase inhibitor, on [^{3}H]thymidine uptake in cells expressing either ErbB3-WT or ErbB3-Y/F (Fig. 6). Cells were treated with

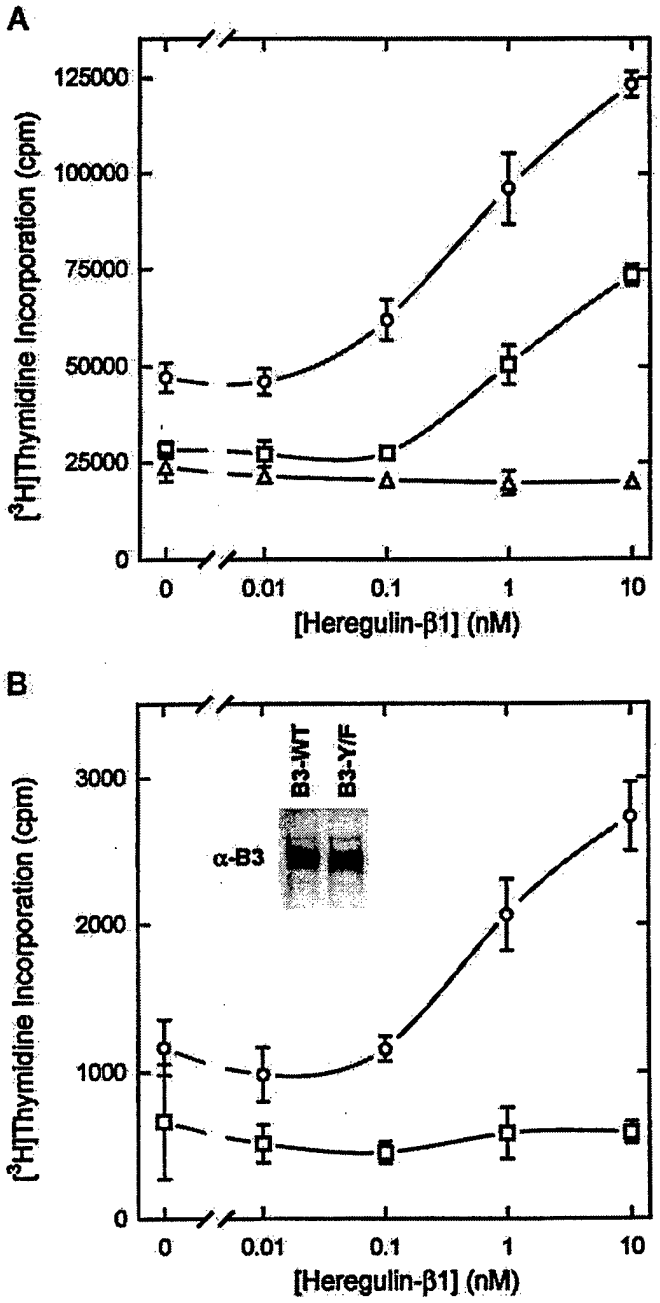

FIG. 5. **Heregulin-stimulated [^{3}H]thymidine uptake by NIH-3T3 cells expressing ErbB3-WT and ErbB3-Y/F.** *A*, NIH-3T3 clones stably transfected with pcDNA3 (△), pcDNA3-B3-WT (○), or pcDNA3-B3-Y/F (□) were serum-starved overnight followed by treatment with varying concentrations of heregulin for 18 h. [*methyl*-^{3}H]thymidine was added to the stimulation medium, and its incorporation into DNA was determined after 4 h. *B*, NIH-3T3 cells transfected with pcDNA3-B3-WT (○), or pcDNA3-B3-Y/F (□) were grown under Geneticin selection for 4 weeks to generate nonclonal pools expressing wild-type and mutant receptors. [^{3}H]Thymidine incorporation was analyzed as in *A*. The *inset* shows expression levels of ErbB3-WT and ErbB3-Y/F in the nonclonal transfected cells as determined by immunoblotting of cell lysates with ErbB3 antibody (*α-B3*). *Error bars* represent the standard deviation of triplicate assays.

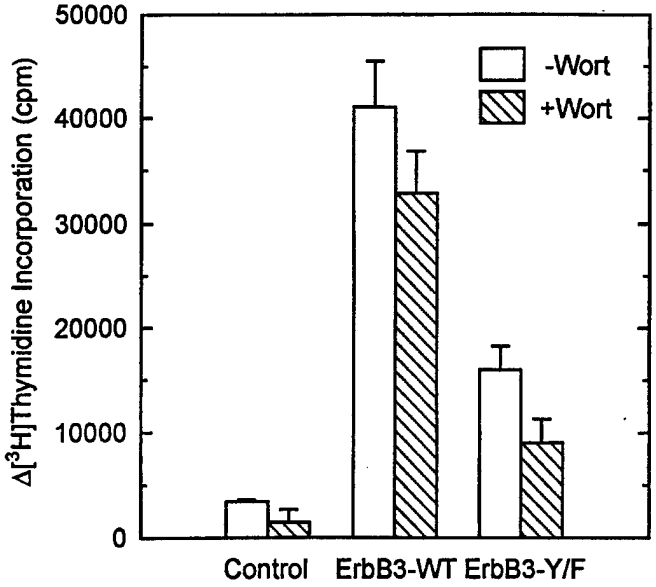

FIG. 6. **Effect of inhibition of PI 3-kinase activity on heregulin-stimulated [³H]thymidine uptake.** Serum-starved cells transfected with pcDNA3, pcDNA3-B3-Y/F, or pcDNA3-B3-WT expression vectors were incubated with wortmannin (*Wort*; 100 nM) or vehicle for 30 min prior to stimulation with either vehicle or heregulin (10 nM) for 18 h. [³H]Thymidine incorporation assays were performed as indicated in Fig. 5. Heregulin-stimulated [³H]thymidine incorporation (Δ[³H]thymidine incorporation) was determined in triplicate assays by subtracting basal incorporation from that stimulated by 10 nM heregulin.

or without wortmannin for 30 min prior to stimulation with either vehicle or 10 nM heregulin. In the representative experiment shown in Fig. 6, heregulin-stimulated DNA synthesis mediated by ErbB3-Y/F was found to be 39% that mediated by ErbB3-WT. Wortmannin decreased heregulin-stimulated [³H]thymidine incorporation in cells expressing ErbB3-Y/F by almost 45% and to a lesser extent (20%) in cells expressing ErbB3-WT. These results implicated PI 3-kinase as another contributor in mitogenic signaling by ErbB2·ErbB3 heregulin coreceptors. A similar effect of wortmannin on heregulin-stimulated DNA synthesis was previously observed in a study of fibroblasts expressing ectopic ErbB2 and ErbB3 proteins (11).

DISCUSSION

The ErbB2 and ErbB3 proteins together constitute a functional heregulin coreceptor (10). Whereas heregulin is a ligand for the ErbB3 receptor protein, ErbB2 does not independently bind heregulin with significant affinity (10, 43), although it may in the context of an ErbB2·ErbB3 heterodimer cooperate in the high affinity binding of heregulin (10). The ErbB3 protein appears to be devoid of intrinsic kinase activity (4, 5). However, C-terminal tyrosine residues of both ErbB2 and ErbB3 are phosphorylated upon stimulation of the ErbB2·ErbB3 coreceptor (10), which is apparently mediated by the protein tyrosine kinase activity of ErbB2 (12). Hence, both ErbB2 and ErbB3, by complementing the functions of one another, can play important roles in heregulin signaling.

Heterodimerization might also increase the diversity of signaling through the activated ErbB2·ErbB3 coreceptor. However, the signal transduction pathways activated by the ErbB2·ErbB3 coreceptor have not been thoroughly characterized. The coupling of PI 3-kinase to ErbB3 in response to heregulin in the ErbB2·ErbB3 coreceptor context (11) and in response to EGF in cells overexpressing EGF receptor and ErbB3 (8, 9) has been documented. The Shc adapter protein has been shown to be phosphorylated in response to heregulin in cells overexpressing both ErbB3 and ErbB4 (40) and in cells overexpressing ErbB4 alone (44). The identification of the potential binding site of Shc on the ErbB3 C terminus by use of peptide competition assays (28) and the heregulin-stimulated ErbB3/Shc association demonstrated in cells expressing the ErbB2 and ErbB3 proteins (7, 40) have implicated Shc in heregulin signal transduction.

The purposes of this study were to demonstrate the binding of Shc to a specific residue in the ErbB3 C terminus in response to heregulin and to determine if this heregulin-induced binding event contributed to the mitogenic response elicited by the ErbB2·ErbB3 coreceptor. We addressed these questions by site-directed mutagenesis of Tyr-1325 in the putative Shc binding site (NPXY motif) (21–24) in the ErbB3 C terminus. Expression of the ErbB3-Y/F mutant protein in NIH-3T3 fibroblasts expressing endogenous ErbB2 resulted in the formation of functional heregulin coreceptors (Fig. 1). Heregulin stimulated the phosphorylation of the mutant ErbB3 protein to a similar extent as the wild-type protein (Fig. 1*A*). However, the Tyr-1325 → Phe substitution abolished interaction of ErbB3 with Shc (Fig. 2*B*), which suggested that Shc specifically bound to phosphorylated Tyr-1325 in the ErbB3 C terminus. The potential of phosphorylated Tyr-1325 of ErbB3 to interact with Shc proteins was subsequently demonstrated by *in vitro* binding experiments (Fig. 2*D*). The observations that heregulin did not (i) stimulate the phosphorylation of Shc (Fig. 2*C*), (ii) stimulate association of Shc with Grb2 (Fig. 2*C*), or (iii) activate MAPK (Fig. 3) in NIH-3T3 cells expressing the ErbB3-Y/F receptor suggested that heregulin-stimulated ErbB3/Shc association was necessary for the activation of these downstream signaling events. Also, it was apparent that any possible interaction of Grb2 with the activated ErbB2 or ErbB3 protein could not effectively activate the Ras/MAPK signaling pathway in the absence of Shc involvement.

Previous studies of NIH-3T3 fibroblasts (29) and T47D mammary carcinoma cells (40) have reported an ErbB2/Shc interaction. In the former study, a chimeric EGF receptor/ErbB2 protein was expressed in NIH-3T3 fibroblasts, and an EGF-dependent association of Shc with the ErbB2 cytoplasmic domain was seen. The latter study of T47D cells documented a heregulin-stimulated ErbB2/Shc association in addition to ErbB3/Shc association. Also, the catalytically activated rat *ErbB2/Neu* oncogene product was found to interact with Shc via an Asn-Leu-Tyr-Tyr (NLYY) sequence motif in the receptor C terminus (20, 45). In contrast, we failed to see any ErbB2/Shc interaction in the NIH-3T3 transfectants in response to heregulin (Fig. 2*B*). One possible explanation for these apparent discrepancies is that in each of these cases phosphorylation of the ErbB2 cytosolic domain occurred in the context of a coreceptor complex with different constituents, which could have resulted in the phosphorylation of distinct subsets of tyrosine residues in the ErbB2 C terminus. In the cases of the chimeric EGF receptor/ErbB2 protein and the *ErbB2/Neu* oncogene product, ErbB2 phosphorylation presumably was mediated by the ErbB2 catalytic domain. In the case of T47D cells, which express all four members of the ErbB family, the ErbB2 protein may have been phosphorylated within a heterodimeric complex with the kinase-active heregulin receptor ErbB4. In the present case, ErbB2 phosphorylation likely occurred in the context of a dimeric complex with the kinase-deficient ErbB3 protein. ErbB2 phosphorylation in this context would require either an intramolecular mechanism (46) or a mechanism involving higher order receptor oligomers (47, 48). Alternatively, our failure to detect ErbB2/Shc association in NIH-3T3 cells overexpressing recombinant ErbB3 in the presence of endogenous ErbB2 might have reflected a relatively low ratio of ErbB2 and ErbB3 protein levels. Indeed, Pinkas-Kramarski *et al.* (7) have

previously demonstrated an ErbB2/Shc association in cells overexpressing both ErbB2 and ErbB3.

Heregulin is mitogenic to a variety of cell types (49) including human mammary cancer cells (2) in which ErbB3 and other members of the ErbB family are often overexpressed. The ErbB2·ErbB3 heterodimeric complex has been shown to be capable of mediating mitogenic and proliferative responses to heregulin, and PI 3-kinase has been shown to be involved in these responses (11). Because the binding of Shc to the ErbB2·ErbB3 coreceptor expressed in our transfected fibroblast cell lines appeared to be directly mediated by the phosphorylation of Tyr-1325 of ErbB3, the Tyr-1325 → Phe mutant ErbB3 protein could be exploited in the investigation of the role of Shc in mitogenic signaling by the ErbB2·ErbB3 heregulin coreceptor.

Whereas heregulin stimulated a dose-dependent increase in DNA synthesis in cells expressing ErbB3-WT, this response was significantly attenuated in cells expressing ErbB3-Y/F (Fig. 5). The high basal mitogenic activity displayed by cells expressing ErbB3-WT was not shown by cells expressing ErbB3-Y/F. Qualitatively similar results were observed when either clonal cells expressing high levels of ErbB3-WT and ErbB3-Y/F or nonclonal pools of cells expressing moderate levels of the ErbB3 proteins were examined, although the residual mitogenic activity of the ErbB3-Y/F protein was enhanced in the clonal cells. The heregulin-stimulated component of DNA synthesis (Δ[^{3}H]thymidine incorporation) in clonal cells expressing ErbB3-Y/F was found to be significantly lower than in clonal cells expressing ErbB3-WT (Fig. 6). The residual mitogenic response to heregulin seen in the cells expressing ErbB3-Y/F could have reflected the activation of the PI 3-kinase pathway (11), which would presumably not be blocked by the Shc binding site mutation. Indeed, ErbB3-Y/F was able to associate with the p85 regulatory subunit of PI 3-kinase to a similar extent as was ErbB3-WT (Fig. 4). Also, pretreatment with the PI 3-kinase inhibitor wortmannin decreased heregulin-stimulated [^{3}H]thymidine uptake in cells expressing ErbB3-Y/F as well as in cells expressing ErbB3-WT (Fig. 6). Whereas the residual mitogenic activity seen in cells expressing ErbB3-Y/F might therefore be attributed in part to the activation of the PI 3-kinase pathway, we conclude that Shc-mediated signaling events contributed significantly to mitogenic signaling by the ErbB2·ErbB3 heregulin coreceptor.

In summary, the results presented in this study indicated that Tyr-1325 in the ErbB3 C terminus is a primary site for the interaction of Shc with the ErbB2·ErbB3 coreceptor complex. Mutation of this tyrosine to phenylalanine abolished association of Shc with ErbB3, blocked activation of the MAPK signaling pathway, and attenuated the mitogenic response to heregulin. Our studies have thus demonstrated that heregulin-induced association of Shc with ErbB3 can initiate signaling events that contribute significantly to the mitogenic effect of heregulin on cells expressing ErbB2·ErbB3 coreceptors.

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Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein

Hong-Hee KIM[1], Ulka VIJAPURKAR, Nathan J. HELLYER, Dolores BRAVO and John G. KOLAND[2]

Department of Pharmacology, The University of Iowa, College of Medicine, Iowa City, IA 52242-1109, U.S.A.

The role of protein tyrosine kinase activity in ErbB3-mediated signal transduction was investigated. ErbB3 was phosphorylated *in vivo* in response to either heregulin (HRG) in cells expressing both ErbB3 and ErbB2, or epidermal growth factor (EGF) in cells expressing both ErbB3 and EGF receptor. A recombinant receptor protein (ErbB3-K/M, in which K/M stands for Lys → Met amino acid substitution) containing an inactivating mutation in the putative ATP-binding site was also phosphorylated in response to HRG and EGF. Both the wild-type ErbB3 and mutant ErbB3-K/M proteins transduced signals to phosphatidylinositol 3-kinase, Shc and mitogen-activated protein kinases. Separate kinase-inactivating mutations in the EGF receptor and ErbB2 proteins abolished ErbB3 phosphorylation and signal transduction activated by EGF and HRG respectively. Hence the protein tyrosine kinase activity necessary for growth factor signalling via the ErbB3 protein seems to be provided by coexpressed EGF and ErbB2 receptor proteins.

INTRODUCTION

The ErbB/HER subfamily of polypeptide growth factor receptors [1,2] comprises the well-characterized epidermal growth factor (EGF) receptor (ErbB1/HER1), the *neu* oncogene product (ErbB2/HER2), and the more recently identified ErbB3/HER3 and ErbB4/HER4 receptor proteins. Each of these receptors is predicted to consist of an extracellular ligand-binding domain, a membrane-spanning domain, a cytosolic protein tyrosine kinase (PTK) domain and a C-terminal phosphorylation domain. The occurrence of heterodimerization among ErbB/HER family receptors seems to enhance the complexity of their cellular signalling mechanisms (see [3]). For example, EGF challenge of cells coexpressing the EGF receptor (EGFR) and either ErbB2, ErbB3 or ErbB4 leads to the phosphorylation of both the EGFR and the associated ErbB family member [3–11]. Various polypeptides in the heregulin (HRG or neuregulin) family have been shown to bind with high affinity to the ErbB4 receptor protein [12,13], but they apparently also bind to ErbB3 [13,14] and an ErbB2/ErbB3 [15] co-receptor complex. Whereas HRG has been shown to activate the PTK activity of the ErbB4 protein [12], it does not seem to activate PTK activity in cells expressing the ErbB3 protein alone [15]. ErbB2 does not itself bind HRG but coexpression of ErbB2 and ErbB3 yields a high-affinity HRG co-receptor, the PTK of which is activated in response to HRG binding [15,16].

Experiments *in vitro* have indicated that the PTK activity of the ErbB3 protein is attenuated significantly relative to that of other ErbB/HER family members; this has been attributed to the occurrence of non-conservative amino acid substitutions in the predicted catalytic domain of ErbB3 [17,18]. However, the ErbB3 protein is known to be strongly phosphorylated in a variety of cellular contexts. For example, ErbB3 is constitutively phosphorylated on tyrosine residues in a subset of human breast cancer cell lines overexpressing this protein [19], and is phosphorylated in response to either EGF or HRG in cells coexpressing ErbB3 and the EGFR [7,8] or ErbB2 [15] respectively. If the ErbB3 protein is devoid of intrinsic PTK activity, its phosphorylation in the cellular context would be presumed to result from the action of an associated PTK, for example via a transphosphorylation by either the EGFR or ErbB2 PTK. However, it is also possible that the PTK of ErbB3 is masked under the conditions of previous experiments *in vitro* or is expressed only in the context of an activated co-receptor complex. Indeed, Kraus et al. [19] observed a ligand-stimulated PTK activity in the ErbB3 protein.

The ErbB3 receptor is unique among receptor PTKs in that the amino acid sequence of the C-terminal phosphorylation domain [20,21] contains six repeats of the motif Tyr-Xaa-Xaa-Met (YXXM), known to serve, when phosphorylated, as a binding site for the Src homology 2 (SH2) domains of the p85 subunit of phosphatidylinositol (PtdIns) 3-kinase [22,23]. In previous studies, chimaeric proteins composed of the extracellular ligand-binding domain of EGFR and the cytoplasmic domain of ErbB3 were used to examine the cellular proteins involved in ErbB3 signal transduction [24,25]. EGF stimulation of NIH-3T3 cells expressing these chimaeric proteins resulted in their increased phosphorylation on tyrosine residues and triggered their association with PtdIns 3-kinase. Subsequent investigations of cancer cells endogenously expressing both the EGFR and ErbB3 [7,8] and transfected fibroblasts ectopically expressing these proteins [26] showed EGF-dependent ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association. As the YXXM motif is not present in the C-terminal phosphorylation domain of the EGFR, it seems that ErbB3 might co-operate with EGFR in the activation of a mitogenic signalling pathway not otherwise directly engaged by the EGFR.

ErbB3, like other ErbB family receptor proteins [27], also incorporates a consensus motif, Asn-Pro-Xaa-Tyr (NPXY), for binding the PTB (phosphotyrosine-binding) domain of the Shc protein [28–32]. This motif has been implicated in the binding of Shc to EGFR and ErbB2 [28,33,34] and to the insulin receptor [35]. Phosphopeptide competition experiments have indicated that the sequence Asn-Pro-Asp-Tyr^{1309} (NPDY) in human ErbB3, when phosphorylated, mediates the association of Shc with this receptor [25], a finding confirmed by our recent site-

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; GST, glutathione S-transferase; HRG, heregulin; K/M, Lys → Met amino acid substitution; MAPK, mitogen-activated protein kinase; PTK, protein tyrosine kinase.

[1] Present address: Chosun University Dental College, Kwangju, Korea.

[2] To whom correspondence should be addressed (e-mail john-koland@uiowa.edu).

directed mutagenesis experiments (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). The Shc protein is rapidly phosphorylated on activation of various receptor or non-receptor PTKs and is involved in the translocation of the Grb2/Sos complex to the plasma membrane [36,37]. Sos, a Ras guanine nucleotide exchange factor, then activates the Ras protein [38,39], which in turn stimulates the phosphorylation and activation of mitogen-activated protein kinases (MAPKs or ERKs) through a protein kinase cascade [40–42].

The present study examined the role of receptor PTK activity in growth factor signal transduction mediated by the ErbB3 protein in conjunction with other ErbB family members. To this end, EGFR, ErbB2 and ErbB3 proteins, either alone or in combination, were expressed ectopically in cultured cell models. The importance of the intrinsic PTK activity of individual ErbB family members was assessed by the use of recombinant EGFR, ErbB2 and ErbB3 proteins incorporating kinase-inactivating mutations.

EXPERIMENTAL

Cell lines and reagents

NIH-3T3 and COS7 cells were purchased from the American Type Culture Collection and cultured as recommended. Recombinant HRG-β1 and antibodies recognizing ErbB2 (Ab-1) and ErbB3 (2F12, 2C3) were purchased from NeoMarkers. Phosphotyrosine-specific monoclonal antibody PY20 was obtained from Leinco Technologies and Transduction Laboratories. Shc, Grb2 and p85 antibodies and recombinant PY20 conjugated with horseradish peroxidase (RC20) were also acquired from Transduction Laboratories. EGFR-specific monoclonal antibody Ab4 and an MAPK-specific antibody were obtained from Oncogene Science and Zymed Laboratories respectively. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were purchased from Amersham. The expression plasmid containing wild-type rat ErbB3 cDNA, pcDNA3-ErbB3, is detailed elsewhere [43]. cDNA species encoding wild-type EGFR and the kinase-deficient EGFR-K/M (in which K/M stands for Lys → Met amino acid substitution) mutant protein were kindly provided by Dr. Gordon Gill (University of California, San Diego, CA, U.S.A.). The rat ErbB2 (Neu) expression plasmid was a gift from Dr. Robert Weinberg (Whitehead Institute, Cambridge, MA, U.S.A.).

Generation of ErbB3 and ErbB2 Lys → Met mutant proteins

Rat ErbB3 cDNA was mutated with the Transformer Site-directed Mutagenesis Kit (Clontech). A Met codon was introduced at amino acid position 740 to replace the Lys codon by using a 24 bp mutagenic primer, 5′-CAGTCTGCATTATGGTCATGGAGG-3′. Lys^{758} of rat ErbB2 cDNA was mutated by use of the Ex-Site Mutagenesis kit (Stratagene) and a 24 bp mutagenic primer, 5′-CATCATGGTGTTGAGAGAAAACA-C-3′. The affected regions of the ErbB2 and ErbB3 cDNA species were sequenced to verify that the mutant expression vectors had been constructed successfully.

Production of recombinant HRG-$\beta1^{177-241}$

The cDNA encoding the EGF-like domain of the β1 isoform of HRG (HRG-$\beta1^{177-241}$) was generated by reverse transcriptase-mediated PCR with the oligonucleotides 5′-GCGAATTCCC-ATCTTGTAAAATGTGCG-3′ (forward primer, corresponding to bp 623–641 of human HRG-β1 cDNA) and 5′-CTCGGCC-GCTACTCCGCCTCCATAAATTCAATC-3′ (reverse primer, corresponding to bp 794–815). The first-strand cDNA was transcribed from human brain mRNA (Clontech) with the reverse primer and SuperscriptII reverse transcriptase (Gibco-BRL). PCR (30 cycles) was performed after addition of the forward primer and *Pfu* polymerase (Stratagene). The authenticity of the PCR-amplified HRG cDNA was verified by DNA sequencing, and the HRG cDNA was subcloned downstream of the glutathione S-transferase (GST) coding sequence in the expression vector pGEX-KG [44]. The GST–HRG fusion protein was purified from cultures of *Escherichia coli* by glutathione–agarose affinity chromatography, dialysed and then digested with thrombin for 30 min at room temperature. The majority of GST and undigested GST–HRG was removed by running the mixture through a second glutathione–agarose column. HRG was freeze-dried and further purified by Superose Fast Performance Liquid Chromatography (Pharmacia). Fractions containing HRG were identified by SDS/PAGE [45], pooled and stored at −85 °C. These manipulations resulted in the inclusion of nine amino acid residues, Gly-Ser-Pro-Gly-Asp-Pro-Pro-Ala-Asn, upstream of the authentic HRG peptide sequence. In some experiments a 30 kDa HRG-β1 protein (NeoMarkers) was employed, which corresponded to the secreted form of the growth factor. Although the potency of this protein was significantly greater than that of the HRG-$\beta1^{177-241}$ peptide, qualitatively similar results were obtained with the two HRG preparations.

Gene transfection, immunoprecipitation and immunoblotting

COS7 cells (10^7) were suspended in Dulbecco's modified Eagle's medium, electroporated at 250 V and 1180 μF (Cell-Porator; Gibco-BRL) in the presence of the indicated plasmids, and used for experiments at 36–60 h after transfection. NIH-3T3 cells were transfected by a calcium phosphate method [46], and clones expressing the ectopic genes were selected in the presence of geneticin (G418).

Dishes of cells (80–90 % confluent) were serum-starved for 16–20 h with cell culture medium containing 0.1 % (v/v) fetal calf serum. Cells were washed twice with serum-free medium and incubated with EGF or HRG diluted in culture medium containing 0.1 % BSA or the dilution vehicle for 5–7 min at 37 °C. Cells were washed twice with PBS and lysed with NP40 lysis buffer [1 % (v/v) Nonidet P40/50 mM Hepes/Na/150 mM NaCl/2 mM EDTA/3 mM EGTA/2 mM sodium orthovanadate/10 mM sodium pyrophosphate/50 mM NaF/2 μg/ml pepstatin A/10 μg/ml aprotinin/10 μg/ml leupeptin/2 mM PMSF (pH 7.4)]. The total cell lysate was centrifuged for 10 min at 13000 *g* and the supernatant was collected. Appropriate antibodies were added and incubated for 1 h on ice. Protein A–agarose or protein G–agarose was added and the suspensions were rocked for 1–3 h at 4 °C. Immunoprecipitates were washed with a buffer containing 1 % (v/v) Nonidet P40, 50 mM Hepes/Na, 150 mM NaCl, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 50 mM NaF, pH 7.4, and subjected to SDS/PAGE. Resolved proteins were transferred to a PVDF membrane and detected with the indicated antibodies by enhanced chemiluminescence luminography (Amersham). For the gel-shift assay of MAPK activation, the amount of bisacrylamide was reduced [acrylamide/bisacrylamide, 30:0.04 (w/v)] and the electrode buffer was used at double concentration.

RESULTS

EGF-dependent phosphorylation of ErbB3 in transfected cells expressing EGFR and ErbB3 proteins

Our previous studies have shown that the ErbB3 protein is an excellent substrate for the PTK activity of the EGFR [18].

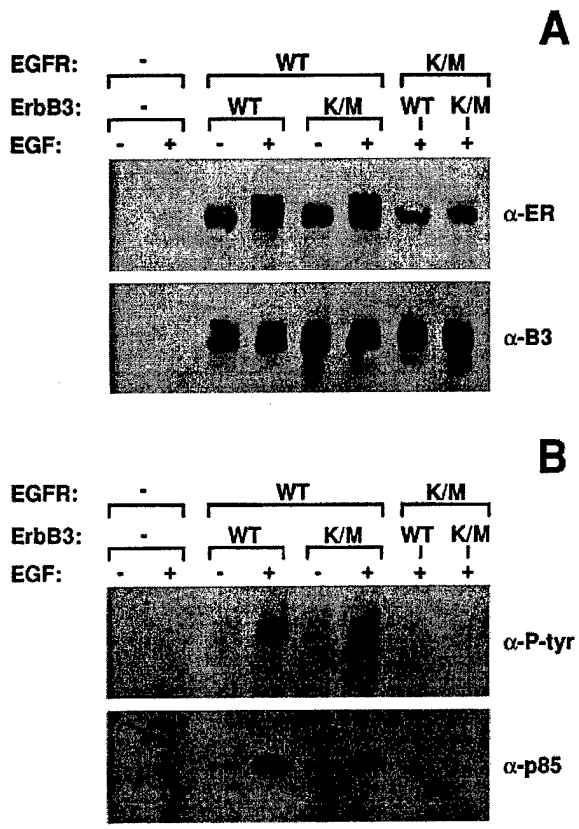

Figure 1 EGF-dependent ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in cells co-transfected with wild-type and mutant EGFR and ErbB3 cDNA species

COS7 cells were transfected by electroporation with wild-type (WT) EGFR, WT ErbB3, Lys^{721} → Met (K/M) mutant EGFR, or Lys^{740} → Met (K/M) mutant ErbB3 cDNA species as indicated. At 24 h after transfection, cells were serum-starved for 11 h and stimulated with 50 nM EGF or vehicle for 5 min at 37 °C. Detergent lysates of the cells were immunoprecipitated with ErbB3-specific antibody. (**A**) Expression of the recombinant ErbB3 protein in transfected cells was demonstrated by subjecting each immunoprecipitate to SDS/PAGE and immunoblotting with ErbB3-specific antibody (α-B3). EGFR expression was shown by immunoblotting an equivalent portion of each cell lysate with EGFR-specific antibody (α-ER). (**B**) Each immunoprecipitate was immunoblotted with anti-phosphotyrosine (α-P-tyr) and an antibody recognizing the p85 subunit of PtdIns 3-kinase (α-p85).

Evidence suggesting that the ErbB3 protein is a physiological substrate for EGFR has also been presented [7,8]. In these studies, cultured cells expressing high levels of both EGFR and ErbB3 were stimulated with EGF; phosphorylation of ErbB3 on tyrosine residues was detected by immunoblotting. To demonstrate more directly the cross-phosphorylation of ErbB3 by the EGFR, COS7 cells were co-transfected with EGFR and ErbB3 expression vectors. Both wild-type and mutant receptor proteins containing a kinase-inactivating Lys → Met substitution in their putative ATP-binding sites (EGFR-K/M [47] and ErbB3-K/M) were employed in these experiments.

Immunoblotting with receptor-specific antibodies indicated that each of the wild-type and K/M mutant receptors was successfully expressed in COS7 cells (Figure 1A). Cell lysates were subsequently immunoprecipitated with ErbB3 antibody; the immunoprecipitates were subjected to immunoblotting with anti-phosphotyrosine. In cells expressing wild-type receptors, EGF stimulated the phosphorylation of the ErbB3 protein (Figure 1B). Significantly, the mutant ErbB3-K/M protein was phosphorylated on tyrosine residues to a similar extent to that of the wild-type protein. The ErbB3 immunoprecipitates showed no presence of EGFR when probed with an EGFR-specific antibody (results not shown). These results demonstrated that intrinsic ErbB3 PTK activity was not necessary for EGF-dependent ErbB3 phosphorylation. Activation of the EGFR also promoted the co-precipitation of p85 with both the wild-type and the mutant ErbB3 proteins (Figure 1B). In contrast, stimulation of the kinase-deficient form of the EGFR (EGFR-K/M) did not induce ErbB3 phosphorylation or ErbB3/p85 association. Therefore the activation of ErbB3 phosphorylation and signal transduction by EGF depended on the PTK activity of the EGFR but did not require that of ErbB3.

The apparent cross-phosphorylation of ErbB3 by the EGFR was not detected in parental COS7 cells, which indicated that the possible presence of endogenous EGFR and ErbB3 proteins in this cell line did not compromise the interpretation of these experiments (Figure 1B). Also, transfected COS7 cells over-expressing the ErbB3 proteins alone showed no phosphorylation of ErbB3 on stimulation with EGF (results not shown). These results were consistent with previous findings that the EGFR-mediated phosphorylation of ErbB3 occurs only in cells expressing a high level of EGFR [7,8].

HRG-dependent ErbB3 phosphorylation and ErbB3 signal transduction in cells expressing wild-type and mutant ErbB3 proteins

To extend our understanding of signal transduction mediated by the HRG-stimulated ErbB3 receptor, we generated stable NIH-3T3 cell lines that expressed a high level of wild-type ErbB3 and ErbB3-K/M (Figure 2A). Cells expressing the wild-type protein showed a constitutive phosphorylation of ErbB3 on tyrosine residues. However, as we have previously observed [43], phosphorylation of ErbB3 was clearly enhanced on stimulation with HRG-$\beta 1^{177-241}$, a recombinant protein comprising the EGF-like domain of the HRG-β1 molecule. The mutant protein ErbB3-K/M was phosphorylated in response to HRG to a similar extent to the wild-type protein, which showed that intrinsic ErbB3 kinase activity was not required for HRG-dependent phosphorylation of the receptor. Treatment of mock-transfected cells with HRG did not elicit any phosphorylation response (Figure 2A). Also, ErbB3 immunoprecipitates showed no presence of the ErbB2 protein when probed with an ErbB2 antibody (results not shown). Given that ErbB3 seems not to possess intrinsic kinase activity [17,18], it was presumed that ErbB2, present endogenously in the NIH-3T3 fibroblasts, mediated the HRG-dependent phosphorylation of ErbB3. Our recent immunoblotting experiments have indicated that NIH-3T3 fibroblasts express the ErbB2 protein (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work), and when anti-phosphotyrosine immunoprecipitates were subjected to immunoblotting with ErbB2 antibody, HRG was seen to augment the phosphorylation of the endogenous ErbB2 protein (Figure 2A). These results suggested that the intrinsic kinase activity of ErbB2 might be responsible for the HRG-dependent phosphorylation of both ErbB2 and ErbB3, which was consistent with the notion that ErbB2 and ErbB3 functioned as HRG co-receptors.

To examine further the signalling potentials of the wild-type and mutant ErbB3 proteins, ErbB3 immunoprecipitates from transfected cells were probed with an antibody recognizing the p85 subunit of PtdIns 3-kinase. An HRG-dependent ErbB3/p85 association was seen with cells expressing either the wild-type or the mutant protein (Figure 2B). Apparently, mutation of the

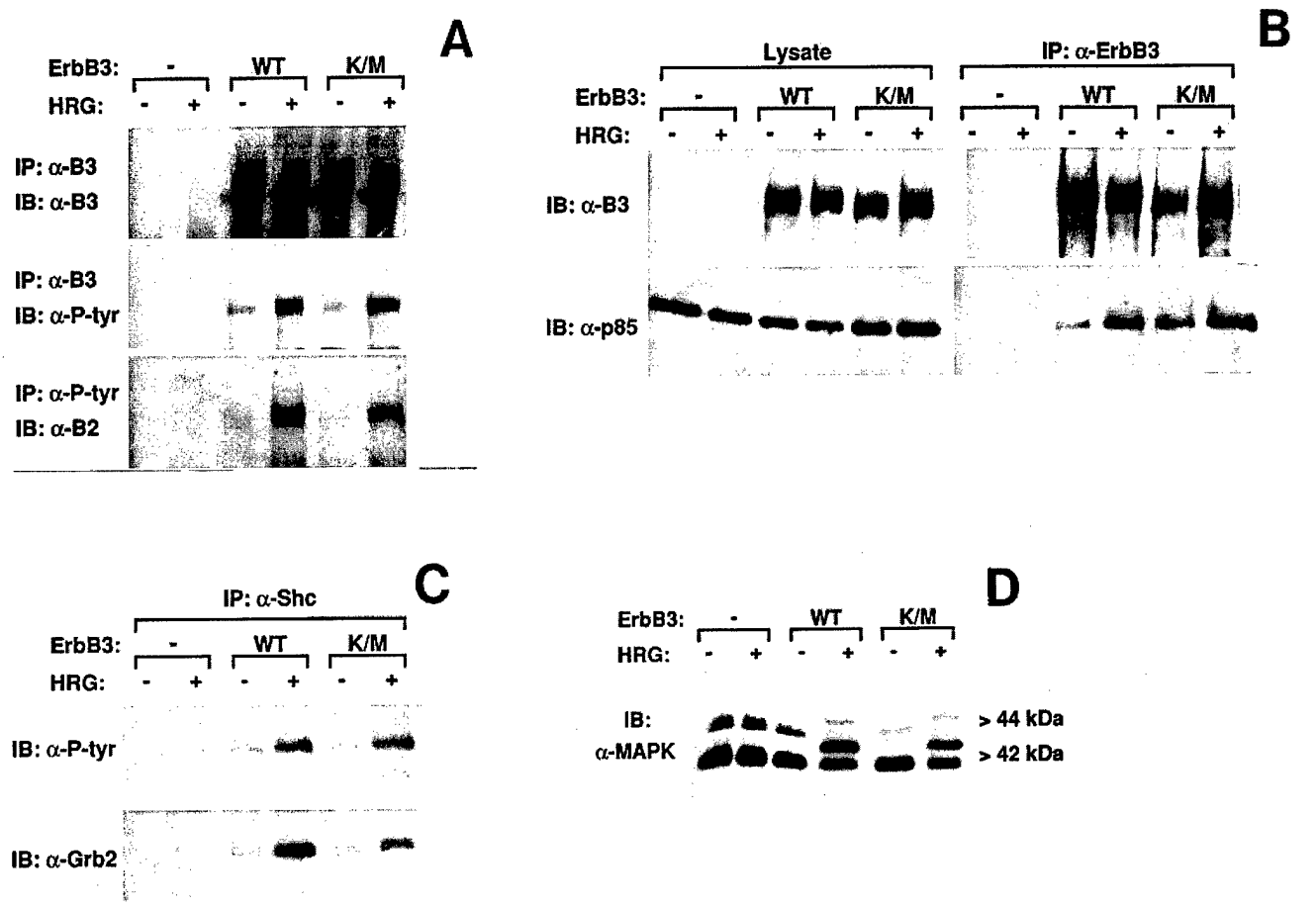

Figure 2 HRG-mediated phosphorylation and signal transduction of wild-type and mutant ErbB3 proteins in stably transfected NIH-3T3 cells

NIH-3T3 cells were transfected with either the parent pcDNA3 expression vector (—) or pcDNA3 vectors incorporating ErbB3-WT or ErbB3-K/M cDNA species as indicated. Cloned cells expressing comparable levels of wild-type (WT) and mutant proteins were selected and propagated. Mock-, ErbB3-WT- and ErbB3-K/M-transfected cells were serum-starved for 16 h and stimulated with either 30 nM HRG-$\beta 1^{177-241}$ or vehicle for 5 min at 37 °C. (**A**) HRG-stimulated phosphorylation of ErbB3-WT, ErbB3-K/M, and endogenous ErbB2 proteins. HRG-stimulated or control cells were dispersed in NP40 lysis buffer. The lysates were immunoprecipitated (IP) with either ErbB3-specific antibody (α-B3) or anti-phosphotyrosine (α-P-tyr), and the immunoprecipitates were resolved by SDS/PAGE. ErbB3 immunoprecipitates were immunoblotted (IB) with either ErbB3 antibody or anti-phosphotyrosine as indicated. Anti-phosphotyrosine immunoprecipitates were immunoblotted with ErbB2-specific antibody (α-B2). (**B**) HRG-stimulated association of the p85 subunit of PtdIns 3-kinase with wild-type and mutant ErbB3 proteins. Lysates of HRG-stimulated or control cells were immunoprecipitated with ErbB3-specific antibody, and the immunoprecipitates were subjected to SDS/PAGE. Immunoprecipitation of the ErbB3 protein and HRG-stimulated co-immunoprecipitation of p85 were detected by immunoblotting with anti-ErbB3 (α-B3) and anti-p85 (α-p85) respectively. (**C**) Stimulation of Shc phosphorylation and Shc/Grb2 association by HRG. Cells treated as in (**A**) were lysed and immunoprecipitated with Shc-specific antibody. Immune complexes were immunoblotted with either the anti-phosphotyrosine–horseradish peroxidase conjugate RC20 (α-P-tyr) or a Grb2-specific antibody (α-Grb2). HRG-stimulated phosphorylation of the 52 kDa isoform of the Shc protein is indicated in the anti-phosphotyrosine blot. (**D**) HRG-stimulated activation of MAPK mediated by wild-type and mutant ErbB3 proteins. Cells were challenged with HRG or vehicle, then lysed as described above. Each cell lysate (30 μg of protein) was subjected to the gel mobility-shift assay of MAPK activation (see the Experimental section). Both the p42 and p44 forms of MAPK were observed in non-stimulated cells. The appearance of more slowly migrating bands indicated the activation of both MAPK forms in response to HRG.

ATP binding site of ErbB3, which would abolish any intrinsic kinase activity, did not affect its ability to associate with the p85 protein, an event presumably mediated by the HRG-dependent phosphorylation of the ErbB3 C-terminus.

The signal-transducing protein Shc has been implicated in the actions of a number of receptor PTKs [30,48–50]. In our recent studies (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work), Shc was found to associate with the ErbB3 protein and was phosphorylated on tyrosine residues in response to HRG. We sought to determine whether the HRG-stimulated kinase-inactive ErbB3-K/M would similarly involve Shc. Cells expressing either wild-type ErbB3 or the ErbB3-K/M mutant protein were stimulated with HRG-$\beta 1^{177-241}$, and Shc immunoprecipitates were blotted with phosphotyrosine antibody. Shc was seen to be phosphorylated in response to HRG in cells expressing either protein (Figure 2C). Negligible Shc phosphorylation was detected in the mock-transfected NIH-3T3 cells. Among the three isoforms of Shc, phosphorylation of p52 was more evident than that of p61 or p46. Anti-Shc immunoprecipitates from the NIH-3T3 transfectants were also probed with a Grb2-specific antibody. HRG-$\beta 1^{177-241}$ seemed to elicit a strong association of Grb2 and Shc in cells expressing ErbB3 (Figure 2C). Although the response of the mutant receptor protein was apparently weaker, ErbB3-K/M also mediated an HRG-dependent Shc/Grb2 association.

In the context of NIH-3T3 cells expressing wild-type ErbB3, MAPK isoforms undergo an HRG-dependent gel mobility shift (presumably owing to phosphorylation by MAPK kinase [51]), which corresponds to the activation of MAPK activity as indicated by immune complex kinase assays (U. Vijapurkar, K.

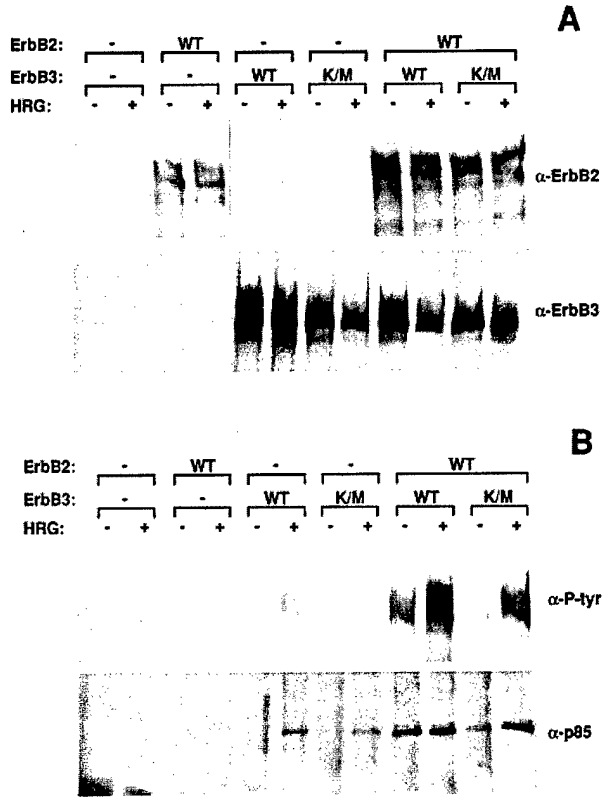

Figure 3 HRG-stimulated ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in COS7 cells transfected with ErbB2 and ErbB3 cDNA species

(**A**) Coexpression of ErbB2 with wild-type (WT) and kinase-deficient ErbB3 proteins. COS7 cells were transiently transfected with the parent expression vector (—), or vectors incorporating cDNA species encoding ErbB2-WT, ErbB3-WT or ErbB3-K/M proteins as indicated. Transfected cells were serum-starved and then stimulated with 30 nM HRG-$\beta 1^{177-241}$ or vehicle for 7 min at 37 °C. Detergent lysates (1 mg of protein) were immunoprecipitated with ErbB3-specific antibody, and the immunoprecipitates were subjected to SDS/PAGE and immunoblotting with ErbB3 antibody (α-B3). The expression of ErbB2 was shown by immunoblotting 10 μg of cell lysate protein with ErbB2-specific antibody (α-B2). (**B**) HRG-stimulated ErbB3 phosphorylation and ErbB3/p85 association in cells expressing wild-type or mutant ErbB2 and ErbB3 proteins. The immunoprecipitates described in (**A**) were resolved by SDS/PAGE, and the phosphorylated forms of wild-type and mutant ErbB3 were detected by immunoblotting with anti-phosphotyrosine (α-P-tyr). Association of the p85 subunit of PtdIns 3-kinase with the ErbB3 proteins was detected by immunoblotting with p85 antibody (α-p85).

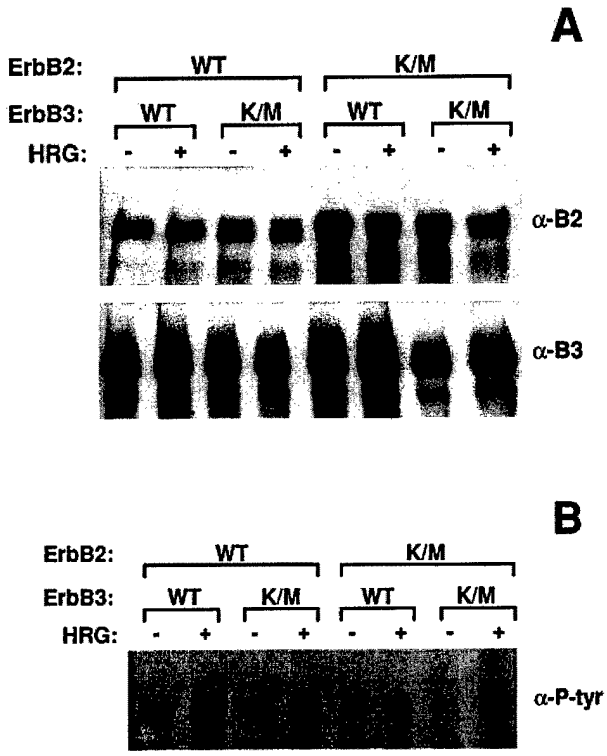

Figure 4 HRG-stimulated ErbB3 phosphorylation in cells co-transfected with wild-type and mutant ErbB2 and ErbB3 cDNA species

(**A**) COS7 cells were transiently transfected with vectors incorporating cDNA species of either wild-type (WT) or kinase-deficient $Lys^{758} \rightarrow$ Met (K/M) ErbB2 and either ErbB3 or ErbB3-K/M proteins as indicated. Cells were serum-starved and then stimulated with 1 nM recombinant HRG-$\beta 1$ (NeoMarkers) for 5 min at 37 °C. Detergent lysates (1.5 mg of protein) were immunoprecipitated with ErbB3 antibody, and each immunoprecipitate was subjected to SDS/PAGE and immunoblotting with ErbB3-specific antibody (α-B3). The expression of ErbB2 was shown by immunoblotting 10 μg of cell lysate protein with ErbB2-specific antibody (α-B2). (**B**) HRG-stimulated ErbB3 phosphorylation in cells coexpressing either ErbB2 or ErbB2-K/M and either ErbB3 or ErbB3-K/M. ErbB3 immunoprecipitates described in (**A**) were resolved by SDS/PAGE and immunoblotted with anti-phosphotyrosine (α-P-tyr).

Cheng and J. G. Koland, unpublished work). The gel mobility-shift assay [52] was used to determine whether HRG could stimulate MAPK phosphorylation via the ErbB3-K/M receptor protein. In the present study, when transfected cells were stimulated with HRG-$\beta 1^{177-241}$, both p42 (Erk2) and p44 (Erk1) forms of MAPK showed retarded migration on SDS/polyacrylamide gels (Figure 2D). With respect to this MAPK gel mobility shift, wild-type and mutant ErbB3 proteins were indistinguishable. The mock-transfected NIH-3T3 cells showed no HRG-dependent MAPK shift. These results confirmed that the Shc/Grb2/MAPK pathway, as well as the PtdIns 3-kinase pathway, was involved in HRG-stimulated signal transduction by ErbB3. Importantly, each of the signal transduction events mediated by the wild-type ErbB3 protein was also mediated by the ErbB3-K/M mutant. These results demonstrated that any intrinsic PTK activity of ErbB3 was not required for these signalling functions.

ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in COS7 cells expressing ErbB2 and ErbB3 receptors

To investigate more directly the role of the ErbB2 receptor PTK in the HRG-stimulated phosphorylation of the ErbB3 protein, wild-type ErbB3 and ErbB3-K/M receptors were expressed alone or together with ErbB2 in COS7 cells (Figure 3A). When the transfected cells were stimulated with HRG, both wild-type and mutant ErbB3 proteins were phosphorylated on tyrosine residues (Figure 3B). ErbB3 phosphorylation was greatly enhanced by co-transfection of ErbB2, which indicated that ErbB3 was phosphorylated by ErbB2 and/or an ErbB2-associated PTK. Again, negligible ErbB2 protein was detected in the ErbB3 immunoprecipitates (results not shown). The weak phosphorylation of the ErbB3 proteins in the cells transfected with ErbB3 cDNA species alone (Figure 3B) might have reflected the activity of a low level of endogenous ErbB2 present in these cells (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). Both in cells transfected with ErbB3 cDNA alone and in cells co-transfected with ErbB2 cDNA, the phosphorylation of the ErbB3-K/M mutant protein was slightly less than that of the wild-type protein (see the Discussion section). ErbB3 immunoprecipitates from the transfected COS7 cells were also probed by

anti-p85 immunoblotting (Figure 3B). Association of the p85 subunit of PtdIns 3-kinase with both wild-type and mutant ErbB3 was found to be dependent on HRG-stimulation and was enhanced by the coexpression of ErbB2.

To underscore further the role of ErbB2 PTK activity in the HRG-stimulated phosphorylation of the ErbB3 protein, ErbB3 and ErbB3-K/M receptors were coexpressed with wild-type or $Lys^{758} \rightarrow Met$ (K/M) mutant ErbB2 receptor (Figure 4A). When coexpressed with the wild-type ErbB2 protein, both wild-type ErbB3 and ErbB3-K/M were phosphorylated on tyrosine residues in response to HRG (Figure 4B). This phosphorylation was almost completely abolished when the wild-type or mutant ErbB3 protein was coexpressed with the ErbB2-K/M protein, which indicated that this phosphorylation was dependent on a functional ErbB2 PTK. The weak HRG-stimulated phosphorylation of ErbB3 and ErbB3-K/M in cells coexpressing the ErbB2-K/M protein was similar to that seen in COS7 cells transfected with ErbB3 cDNA species alone (compare Figure 3B).

DISCUSSION

The EGF-stimulated phosphorylation of ErbB3 has been demonstrated in studies of human cancer cell lines endogenously expressing EGFR and ErbB3 [7,8] and cultured cells ectopically expressing these receptors [11]. This apparent receptor cross-phosphorylation was confirmed here in studies of COS7 cells co-transfected with EGFR and ErbB3 cDNA species (Figure 1). Although it is possible that this phosphorylation was mediated by another protein kinase activated by the EGFR, the following observations suggest that the ErbB3 protein was phosphorylated directly by the EGFR. First, the kinase-deficient EGFR protein could not deliver this effect (Figure 1). Secondly, the cytoplasmic domain and C-terminal sequences of ErbB3 have previously been found to be excellent substrates for the EGFR [18,53]. Thirdly, evidence for cross-phosphorylation between other ErbB family receptors has accumulated [3–11,15,54–56].

The potential ErbB2-mediated phosphorylation of the ErbB3 protein was also investigated in the present study. The HRG-stimulated phosphorylation of ErbB3 expressed in COS7 cells was greatly enhanced with the coexpression of ErbB2 (Figure 3; see also [15]), and this effect was also observed with the ErbB3-K/M mutant protein. Coexpression of the kinase-deficient ErbB2-K/M protein with ErbB3 did not, however, significantly enhance HRG-dependent ErbB3 phosphorylation. These results indicated that ErbB3 kinase activity was not required for HRG-dependent ErbB3 phosphorylation, and that the PTK activity of ErbB2 was responsible for this phosphorylation. ErbB2/ErbB3 cross-phosphorylation would provide a mechanism by which both receptors could have important roles in HRG signalling. Whereas ErbB2 has intrinsic kinase activity, it cannot bind HRG in the absence of ErbB3 [15]. In contrast, ErbB3 binds HRG, but apparently does not possess intrinsic enzymic activity [17,18].

The phosphorylation of the ErbB3-K/M mutant protein on tyrosine residues was slightly lower than that of the wild-type protein when transfected COS7 cells were stimulated with HRG (Figures 3 and 4). This might suggest that ErbB3 does have intrinsic kinase activity and that the observed phosphorylation of ErbB3 was the sum of autophosphorylation and cross-phosphorylation by ErbB2. However, as we and others have not detected PTK activity in the ErbB3 protein [17,18], this possibility seems unlikely. Even if the ErbB3 receptor possessed protein kinase activity, the cross-phosphorylating activity of ErbB2 would seem to predominate over this activity, as the presence of ErbB2 greatly enhanced the phosphorylation of ErbB3. An alternative explanation for the weaker HRG-dependent phosphorylation of the ErbB3-K/M mutant is the possibility that the Lys → Met substitution altered the conformation of the mutant protein such that it was less efficiently phosphorylated by ErbB2. Interestingly, no difference between the phosphorylation of ErbB3 wild-type and ErbB3-K/M was detected when the EGFR PTK mediated the cross-phosphorylation process (Figure 1).

The recruitment by ErbB3 of PtdIns 3-kinase to both EGF and HRG signalling pathways was also investigated here (Figures 1, 2 and 3). Both wild-type ErbB3 and the ErbB3-K/M mutant protein were shown to associate with the p85 subunit of PtdIns 3-kinase in a ligand-dependent manner. The presence of several YXXM motifs for p85 binding is unique to ErbB3, *Drosophila* insulin receptor [57] and the IRS-1 protein [58]; like IRS-1, ErbB3 seemed to function as a bridge between a receptor PTK and PtdIns 3-kinase. Previous studies have shown the HRG-dependent activation of PtdIns 3-kinase in NIH-3T3 cells transfected with both ErbB2 and ErbB3 cDNA species [26] and in cultured breast cancer cells endogenously expressing ErbB2 and ErbB3 [59].

At least one NPXY motif for Shc binding is present in each ErbB family protein. Graus-Porta et al. [60] reported that HRG induced the recruitment of Shc to ErbB family receptors and stimulated the phosphorylation of Shc on tyrosine residues in cells overexpressing both ErbB3 and ErbB4. HRG-stimulated phosphorylation of Shc has also been shown in cells overexpressing ErbB4 alone [61]. We have recently shown that ErbB3 can recruit the Shc protein on stimulation by HRG. This recruitment leads to the phosphorylation of Shc on tyrosine residues, the strong association of Shc and Grb2, and the activation of MAPKs (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). Here we have demonstrated that the ErbB3-K/M mutant protein was also capable of mediating these signal transduction events. These results therefore suggest that any intrinsic PTK activity of ErbB3 is not necessary for its signalling function and that ErbB3, when phosphorylated in the presence of a kinase-active ErbB2 protein, can activate at least two distinct signalling pathways.

In summary, we have examined the role of PTK activity in growth factor signal transduction mediated by the ErbB3 protein. Whereas previous studies have shown that this protein exhibits negligible intrinsic PTK activity *in vitro* ([17,18], but see also [19]), the present results indicated that ErbB3 is catalytically inactive even in the context of a co-receptor complex. The ErbB3 protein is apparently phosphorylated by either the EGFR or ErbB2 PTK on stimulation of EGFR/ErbB3 or ErbB2/ErbB3 co-receptors with EGF or HRG respectively. Although the ErbB3 protein must rely on the PTK activity of another ErbB family member, it does uniquely participate in growth factor signalling by providing a high-affinity ligand-binding site in the case of the ErbB2/ErbB3 HRG co-receptor and by recruiting diverse intracellular signalling molecules. Phosphorylation of ErbB3 by a co-receptor partner triggers ErbB3/PtdIns 3-kinase association, Shc/Grb2 association and the activation of MAPKs. Given that the ErbB3 receptor protein is, along with other ErbB family members, often abundantly expressed in human cancer cells, the mitogenic signal transduction mechanisms investigated here might be particularly relevant in this context.

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ErbB3 (HER3) interaction with the p85 regulatory subunit of phosphoinositide 3-kinase

Nathan J. HELLYER, Kunrong CHENG[1] and John G. KOLAND[2]
Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA 52242, U.S.A.

ErbB3 (HER3), a unique member of the ErbB receptor family, lacks intrinsic protein tyrosine kinase activity and contains six Tyr-Xaa-Xaa-Met (YXXM) consensus binding sites for the SH2 domains of the p85 regulatory subunit of phosphoinositide 3-kinase. ErbB3 also has a proline-rich sequence that forms a consensus binding site for the SH3 domain of p85. Here we have investigated the interacting domains of ErbB3 and p85 by a unique application of the yeast two-hybrid system. A chimaeric ErbB3 molecule containing the epidermal growth factor receptor protein tyrosine kinase domain was developed so that the C-terminal domain of ErbB3 could become phosphorylated in the yeast system. We also generated several ErbB3 deletion and Tyr → Phe site-specific mutants, and observed that a single ErbB3 YXXM motif was necessary and sufficient for the association of ErbB3 with p85. The incorporation of multiple YXXM motifs into the ErbB3 C-terminus enabled a stronger ErbB3/p85 interaction. The proline-rich region of ErbB3 was not necessary for interaction with p85. However, either deletion or mutation of the p85 SH3 domain decreased the observed ErbB3/p85 association. Additionally an ErbB3/p85 SH3 domain interaction was detected by an assay *in vitro*. These results were consistent with a model in which pairs of phosphorylated ErbB3 YXXM motifs co-operate in binding to the tandem SH2 domains of p85. Although a contributing role for the p85 SH3 domain was suggested, the N- and C-terminal SH2 domains seemed to be primarily responsible for the high-affinity association of p85 and ErbB3.

INTRODUCTION

ErbB (HER) family receptors regulate mammalian cell survival, proliferation and differentiation in response to growth factors such as epidermal growth factor (EGF) and heregulin [1,2]. ErbB3 (HER3) is unique among ErbB family members in that it is devoid of intrinsic protein tyrosine kinase activity [3,4] as well as being the only family member that contains multiple consensus sites in its C-terminal domain for binding the p85 subunit of phosphoinositide (PI) 3-kinase. Although ErbB3 lacks kinase activity, Tyr residues within its cytoplasmic tail become phosphorylated on its heterodimerization with other ErbB family receptors, such as the EGF receptor (EGFR) or ErbB2, which allows ErbB3 to associate with PI 3-kinase. The EGF-dependent phosphorylation of ErbB3 triggers ErbB3/p85 association and increases the level of PI 3-kinase activity that is co-immunoprecipitated with ErbB3 [5]. Likewise, when ErbB3 is phosphorylated by EGFR *in vitro* and added to cell lysates, ErbB3 co-immunoprecipitates with both p85 and the catalytic activity of the p110 subunit of PI 3-kinase [6]. Additionally it has been shown that in mouse fibroblasts transfected with ErbB2 and ErbB3 cDNA species, PI 3-kinase activity and mitogenesis increase in a heregulin-dependent manner, with both responses being dependent on the presence of ErbB3 [7]. PI 3-kinase activation by the ErbB2/ErbB3 co-receptor complex might also mediate heregulin-induced proliferation in mammary epithelial cells [8].

The p85 regulatory subunit of PI 3-kinase contains five distinct domains: an SH3 domain, a Bcr-homology region, two SH2 domains, and an inter-SH2 (IS) domain. The IS domain has been shown to be the region of p85 responsible for high-affinity interaction with the p110 catalytic subunit of PI 3-kinase [9–13]. The proline-rich Bcr domain exhibits sequence similarity with the breakpoint cluster region gene product [14] and has been implicated in an intramolecular interaction with the p85 SH3 domain [15], as well as in mediating intermolecular associations with the SH3 domains of Src protein tyrosine kinase family members [16–19].

The amino acid sequence motif Tyr-Xaa-Xaa-Met (YXXM) has been shown, when phosphorylated, to specifically associate with the SH2 domains of p85 [20,21]. Six such consensus binding sites for the SH2 domains of p85 occur at the C-terminus of ErbB3; if phosphorylated, they could potentially mediate the association of ErbB3 and p85. Synthetic ErbB3-derived phosphopeptides that contain phosphorylated YXXM sequences have been shown to inhibit the association of p85 with ErbB3 in the context of a chimaeric EGFR/ErbB3 receptor [22]. However, it is not known which specific ErbB3 YXXM motifs are phosphorylated *in vivo*, and whether the C-terminal, the N-terminal, or both p85 SH2 domains account for p85 association with ErbB3.

The C-terminal tail of ErbB3 also contains a proline-rich sequence, PPRPPRP, that forms a consensus binding site for the SH3 domains of both p85 and Src-family protein tyrosine kinases [23–25] and could also contribute to the association of ErbB3 with p85. SH3 domains recognizing PXXP amino acid motifs in

Abbreviations used: cSH2, C-terminal p85 SH2 domain; EGF, epidermal growth factor; EGFR, EGF receptor; ER/B3, chimaeric EGFR/ErbB3 construct; GST, glutathione S-transferase; IS, inter-SH2 domain; n/cSH2, contiguous nSH2, IS and cSH2 domains; nSH2, N-terminal p85 SH2 domain; PI, phosphoinositide.

[1] Present address: Cardiovascular Institute, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, U.S.A.
[2] To whom correspondence should be addressed (e-mail john-koland@uiowa.edu).

signalling proteins have been shown to mediate protein–protein interactions independently as well as in co-operation with SH2 domains. Interestingly, it has been observed that the SH3 domain of p85 can interact with either of two proline-rich motifs [PPTPKPRPPRPLP (residues 84–96) and PAPALPPKPPKP (residues 303–314)] contained within the Bcr region of p85 itself [15]. This intramolecular interaction could cause conformational changes within p85 that regulate its association with other proteins or modulate the catalytic activity of the associated p110 catalytic subunit of PI 3-kinase [15].

The yeast two-hybrid system is a genetic approach for studying protein–protein interactions [26], which has been used to study the interaction between p85 and the insulin and insulin-like growth factor 1 receptors [27,28] as well as the interaction between the p85 and p110 subunits of PI 3-kinase [10]. Here we describe a novel application of the yeast two-hybrid system in mapping the interacting domains of p85 and ErbB3.

EXPERIMENTAL

Yeast strains and expression plasmids

The yeast two-hybrid system reporter strain SFY526 (MATα ura3-52 his3-Δ200 ade2-801 trp-901 leu2-3,112 can^r gal4-542 gal 80-538 URA3::GAL1-lacZ) and the respective Gal4 DNA-binding domain (pGBT9) and transcriptional activation domain (pGAD424) vectors have been described [26]. Various pGAD-p85 vectors [10] were kindly provided by the laboratory of Dr. Jeffrey Pessin (University of Iowa, Iowa City, IA, U.S.A.). The rat ErbB3 cDNA sequence has been described previously [29]. The cDNA encoding the protein tyrosine kinase domain of the human EGFR (amino acid residues 647–972) was excised from pMMTV-ER [30] and subcloned into the yeast two-hybrid expression vector pGBT9 to yield pGBT-EGFRΔCT. The cDNA encoding the C-terminal tail of ErbB3 (residues 938–1339) was subsequently subcloned in-frame with the EGFR protein tyrosine kinase domain sequence by use of an *Acc*I restriction site conserved in both EGFR and ErbB3 cDNA sequences to generate pGBT-ER/B3. pGBT-ER(K/M)/B3 was generated by subcloning into pGBT-ER/B3 a cDNA encoding an EGFR protein tyrosine kinase domain with a Lys-721 → Met mutation that abolished kinase activity [31]. pGBT-ER/B3Δ1121 was made by a *Sac*II/*Sal*I restriction digestion and subsequent religation of the plasmid. pGBT-ER/B3Δ1204 and pGBT-ER/B3Δ1237 were made by PCR amplification of truncated sequences by using the forward primer 5′-ATATATAGCGAGATCTCCTCC-3′ and the reverse primers 5′-ATGTCGACGCTACCCCTTCTCTT-3′ or 5′-ATGTCGACTCTCTGTGTACTGCC-3′ respectively. These primers incorporated *Bgl*II and *Sal*I restriction sites into the amplified sequences that allowed them to be subcloned in-frame with the pGBT-ER/B3 protein tyrosine kinase domain plasmid. The pGBT vector conveniently provides in-frame stop codons for all three deletion mutants. The pGBT-ER/B3-ΔPro, -1051Y, -1194Y, -1051/1194Y, -1051/1194F, -6F and pGAD-p85-D21N site mutants were generated bu using the ExSite mutagenesis method (Stratagene, La Jolla, CA, U.S.A.).

β-Galactosidase activity assays

Quantitative assays of β-galactosidase activity were performed as described by Fields and Song [26], except that Chlorophenol Red β-D-galactopyranoside (Sigma) was used as the chromogenic substrate and the absorbance was determined at 574 nm. One unit of β-galactosidase activity was defined as $(1000 \times A_{574})/[A_{600} \times \text{volume (ml)} \times \text{time (min)}]$ as described by Miller [32]. All results are expressed as means ± S.E.M. for at least four independent assays performed on distinct colonies.

Immunoblotting

Yeast cells were grown in 50 ml of selective medium overnight; they were then pelleted, washed in 1 ml of water and resuspended in 250 μl of 20 % (w/v) trichloroacetic acid. Glass beads (500 μm) were added to the samples, which were then vortex-mixed six times for 30 s each time. The trichloroacetic acid precipitates were neutralized with 250 μl of acetone/ammonium hydroxide (5:0.3, v/v), after which 400 μl of 1 % (w/v) SDS was added and the samples were boiled for 7 min. Lysates were transferred to fresh tubes containing 100 μl of 5 × sample buffer, and 50 μl of each sample was subjected to SDS/PAGE. Resolved proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.), which was blocked with 5 % (w/v) dry milk in PBS then immunostained with ErbB3-specific (2C3; Neo-Markers, Fremont, CA, U.S.A.), GAL4(DBD)-specific (RK5C1, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or p85 N-terminal SH2 domain-specific (Upstate Biotechnology, Lake Placid, NY, U.S.A.) antibodies. Membranes were incubated with horseradish peroxidase-conjugated anti-(mouse Ig) and detected by an enhanced chemiluminescence method (Amersham, Chicago, IL, U.S.A.).

Binding assays *in vitro*

Several cDNA species encoding N-terminal domains of p85 were amplified by PCR and expressed as glutathione S-transferase (GST) fusion proteins, which were purified as described previously [33]. An Asp-21 → Asn amino acid substitution was introduced into the SH3 domain by use of the ExSite mutagenesis method (Stratagene). Each GST or GST–p85 peptide (2 nmol) was incubated in buffer A [20 mM sodium Hepes (pH 7.4)/ 50 mM NaCl/10 % (v/v) glycerol] supplemented with 1.8 mM $MnCl_2$, 0.02 % (w/v) Triton X-100, and 2.0 pmol of purified recombinant ErbB3 cytosolic domain (hisTKD-B3) [4] for 15 min on ice (total volume 200 μl). The mixture was added to glutathione–agarose (1:1 suspension in buffer A) and incubated for 15 min at 4 °C. The agarose suspensions were centrifuged for 5 s at 600 *g*. The pellets were washed in 500 μl of ice-cold buffer A and then suspended in 110 μl of SDS/PAGE sample buffer. Samples (20 μl) were resolved by SDS/PAGE and subjected to immunoblotting with ErbB3 antibody. GST–p85 peptides (910 pmol) were also analysed by SDS/PAGE with Coomassie Blue staining.

RESULTS

Generation of a chimaeric yeast two-hybrid construct to examine the interaction between ErbB3 and p85

Analysis of the deduced amino acid sequence of the ErbB3 C-terminal phosphorylation domain reveals six consensus binding sites for the N-terminal and C-terminal SH2 domains of p85, as well as a proline-rich candidate ligand for the p85 SH3 domain. To examine the interaction between p85 and ErbB3 with the two-hybrid system, we generated a pGBT9 yeast two-hybrid vector expressing the cytosolic domain of ErbB3 (residues 660–1339). The ErbB3 cytosolic domain did not seem to interact with p85 (Figure 1), presumably owing to the lack of intrinsic kinase activity in ErbB3 and the absence of an associating kinase capable of phosphorylating ErbB3 in yeast. To overcome the

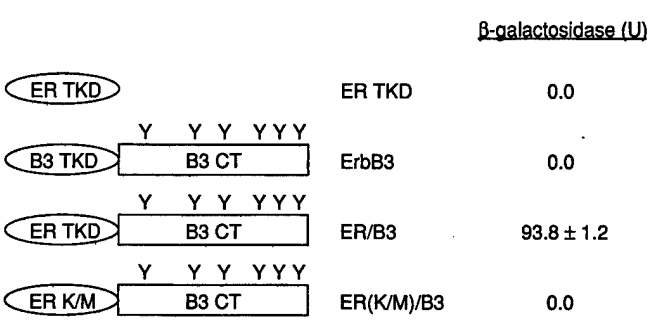

Figure 1 Interaction of an EGFR/ErbB3 chimaeric construct (ER/B3) with the p85 protein in the yeast two-hybrid system

The yeast reporter strain SFY526 was co-transformed with the indicated pGBT-receptor and pGAD-p85 plasmid constructs, and transformants were assayed for β-galactosidase reporter gene activity. The EGFR protein tyrosine kinase domain (ER TKD, residues 647–972) and ErbB3 cytosolic domain (residues 660–1339) are shown schematically. ER/B3 is a chimaera composed of the EGFR protein tyrosine kinase domain (residues 647–918) fused to the phosphorylation domain of the ErbB3 protein (residues 938–1339) and expressed via the pGBT vector. A conserved Lys residue in the EGFR protein tyrosine kinase domain essential for protein kinase activity was replaced with Met in the ER(K/M)/B3 construct. The pGAD-p85 construct expresses the full-length p85 subunit of PI 3-kinase as a GAD fusion protein [10]. Abbreviation: U, units.

lack of phosphorylation in the yeast system, we developed an EGFR/ErbB3 chimaeric construct (ER/B3) containing a functional EGFR protein tyrosine kinase domain in place of the inactive ErbB3 protein tyrosine kinase homology domain. In this construct, the ErbB3 C-terminus was fused to the end of the EGFR protein tyrosine kinase domain in a manner that preserved the conserved amino acid sequences at the domain boundary. By itself, the EGFR kinase domain (ER TKD, residues 647–972) was incapable of association with p85 (Figure 1). However, ER/B3 strongly associated with p85, apparently in a phosphorylation-dependent manner, as a kinase-deficient ER/B3 construct, ER(K/M)/B3, failed to interact with p85 (Figure 1).

To ensure that ER/B3 could not activate the *lacZ* reporter gene independently of p85, ER/B3 was transfected both alone and with a pGAD vector not containing a p85 cDNA insert. No activation of the lacZ reporter gene was observed in either case (results not shown). Similarly, the pGAD-p85 construct did not activate the reporter gene alone or in combination with an empty pGBT vector (results not shown). Western immunoblotting revealed that the GBT-fusion proteins were expressed at similar levels (see Figure 4A), which suggested that differences in protein interactions with p85 were not due to differences in protein expression levels. Taken together, these results demonstrated a specific interaction between ErbB3 and p85 within the yeast two-hybrid system.

Interaction between ER/B3 and p85 subdomains

To determine which regions of p85 associated with ErbB3, we tested the interaction of the SH3, Bcr and dual SH2 domains (n/cSH2) of p85 with ER/B3. The n/cSH2 domain exhibited the strongest interaction of the three domains. However, this interaction was less than half of that of full-length p85 (Figure 2). The Bcr and SH3 domains of p85 seemed to associate very weakly with ER/B3. Even though the SH3 domain of p85 exhibited a minimal interaction when tested alone, we suspected that the SH3 of p85 domain might be capable of a weak interaction with a proline-rich motif in ErbB3 and contribute to the maximally observed interaction between p85 and ErbB3, which seemed to be mediated predominantly by the two SH2 domains of p85. To test this hypothesis we generated a mutant p85 protein (p85-D21N) with an Asp → Asn substitution at Asp-21, which occurs within the SH3 domain of p85. Other groups have shown that this Asp → Asn mutation in the p85 SH3 domain disrupts the specific association between the SH3 domain and proline-rich recognition sequences [24,25]. We found that

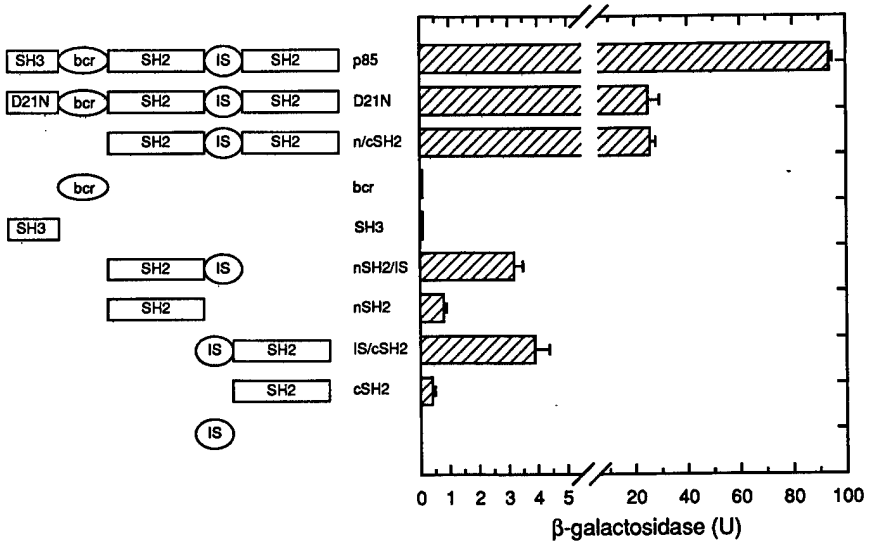

Figure 2 Interaction between ErbB3 and domains of the p85 protein

The yeast reporter strain SFY526 was co-transformed with pGBT-ER/B3 and the indicated pGAD-p85 plasmid constructs. p85-D21N has an Asp-21 → Asn substitution in the SH3 domain of p85. Distinct constructs expressing individual p85 domains as GAD fusion proteins are indicated: n/cSH2 (residues 330–720), Bcr (residues 77–353), SH3 (residues 9–87), nSH2/IS (residues 330–621), nSH2 (residues 330–434), IS/cSH2 (residues 429–720), cSH2 (residues 621–720), and IS (residues 429–621). Abbreviation: U, units.

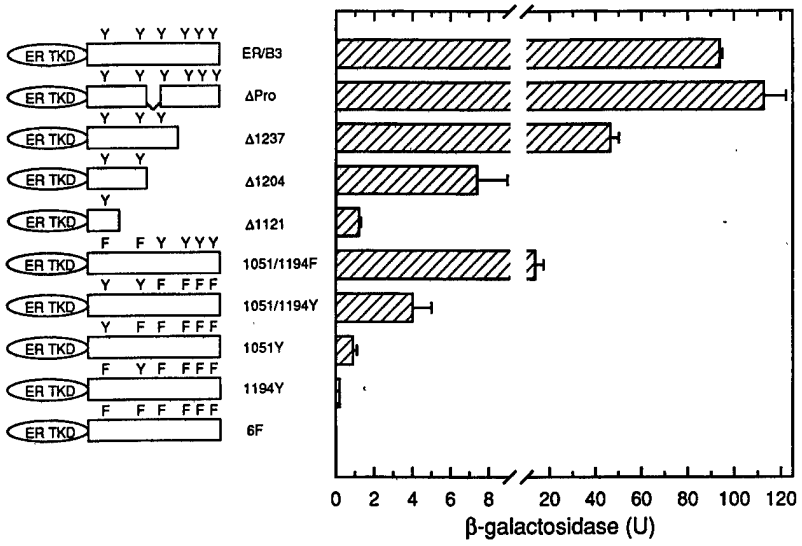

Figure 3 Interaction of C-terminal ER/B3 truncation mutants and Tyr → Phe site-specific mutants with p85

The yeast reporter strain SFY526 was co-transformed with pGAD-p85 and the indicated pGBT-ER/B3 plasmid constructs. Various ER/B3 C-terminal truncation mutants are shown with remaining YXXM motifs indicated. Various ER/B3 site-specific mutants are shown with indicated Tyr → Phe substitutions occurring in consensus sequences (YXXM → FXXM). ER/B3ΔPro corresponds to the deletion of a proline-rich consensus p85 SH3 domain-binding motif (residues 1205-1211). Abbreviation: U, units.

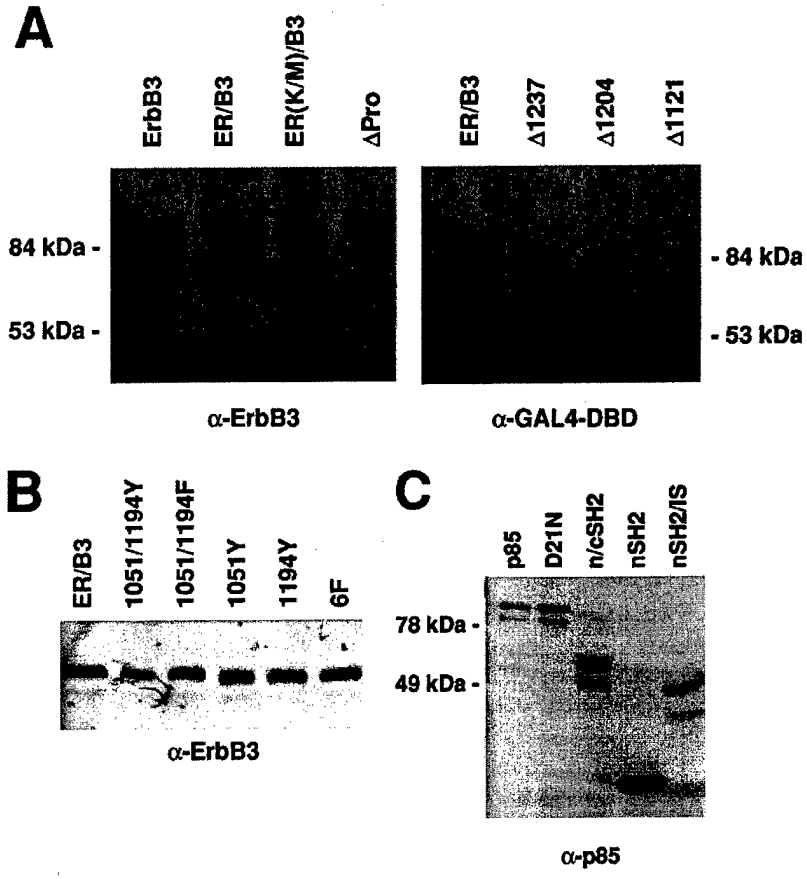

Figure 4 Immunoblotting of GBT and GAD fusion proteins

Lysates of yeast transfected with pGBT and pGAD expression vectors were immunoblotted to detect the expression of the yeast two-hybrid fusion proteins. (**A**) Left panel: detection of the approx. 90 kDa GBT-fusion proteins with an antibody recognizing the C-terminus of ErbB3. Right panel: detection of wild-type GBT-ErbB3 (approx. 90 kDa) and ER/B3 truncation mutants (approx. 75, 65 and 50 kDa) with an antibody specific for the Gal4 DNA-binding domain. (**B**) Detection of ER/B3 Tyr → Phe site mutants. (**C**) Detection of GAD-p85 constructs with an antibody recognizing the N-terminal SH2 domain of p85.

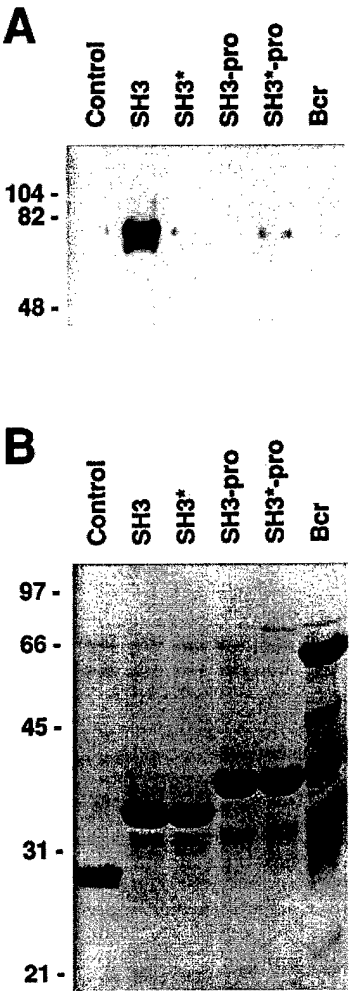

Figure 5 Association *in vitro* of GST–p85-SH3 domain fusion proteins with ErbB3

To investigate the role of the p85 SH3 domain in ErbB3/p85 interactions, GST fusion proteins containing either the p85 SH3 domain (GST–SH3, residues 1–80), the mutant SH3-D21N domain (GST–SH3*), the SH3 domain with a small proline-rich region of the Bcr domain appended (GST–SH3-pro, residues 1–101), or the contiguous SH3 and Bcr domains (GST–SH3-bcr, residues 1–339) were generated. (**A**) GST–p85 fusion proteins (2.0 nmol of each) were incubated with a recombinant ErbB3 cytosolic domain protein (2.0 pmol) and samples of glutathione-agarose precipitates were subsequently resolved by SDS/PAGE. Association of the ErbB3 protein with precipitated GST or GST–p85 fusion proteins was detected by immunoblotting with an ErbB3-specific antibody. (**B**) GST and GST–p85 fusion proteins (0.9 nmol of each) analysed by SDS/PAGE with Coomassie Blue staining.

p85-D21N exhibited a weaker association than wild-type p85 with ER/B3. Interestingly, the p85-D21N and n/cSH2 domain associations with the ER/B3 construct were comparable. The p85-D21N and n/cSH2 domain proteins were expressed at a level similar to or higher than wild-type p85 (see Figure 4C). These results suggested that the tandem SH2 domains of p85 are predominantly responsible for the observed ErbB3/p85 interaction but also suggested that the SH3 domain might have a role in the association of p85 with ErbB3.

Association of the N-terminal and C-terminal SH2 domains of p85 with ER/B3

We next tested whether both SH2 domains were required for the binding of p85 to ErbB3. The individual N-terminal (nSH2) and C-terminal (cSH2) SH2 domains were capable of binding to ErbB3, with or without the IS domain (Figure 2). Inclusion of the IS domain increased the interaction between either nSH2 or cSH2 and ErbB3, perhaps by altering the conformation of the SH2 domains, as the IS domain alone did not interact with ErbB3 (Figure 2). The independent SH2 domains, however, bound much more weakly to ER/B3 than the dual SH2 domain, although comparable expression of these proteins was observed (see Figure 4C). This suggested that the tandem SH2 domains might participate in the ErbB3/p85 interaction via multiple ErbB3 phosphorylation sites.

Role of the ErbB3 proline-rich sequence in the association of p85

To test whether the proline-rich sequence motif in ErbB3 interacted with the SH3 domain of p85, a mutant ER/B3 construct (ER/B3ΔPro) was generated that lacked the PPRPPRP sequence (residues 1205 to 1211). Surprisingly, ER/B3ΔPro exhibited a slightly stronger interaction than ER/B3 with p85 (Figure 3). This result indicated that any potential interaction of the p85 SH3 domain with the PPRPPRP sequence did not significantly enhance the affinity of the ErbB3/p85 interaction. It is possible that the SH3 domain interacted with a sequence of ErbB3 other than the PPRPPRP sequence deleted. Alternatively, a competing intramolecular association of the SH3 domain with proline-rich sequences within the Bcr domain of p85 might have neutralized the effect of an ErbB3/SH3 interaction (see the Discussion section).

Association of p85 with ER/B3 C-terminally truncated mutants and Tyr → Phe site-specific mutants

We also generated several ER/B3-derived constructs with truncations of the ErbB3 C-terminal domain and the corresponding loss of YXXM motifs from the ErbB3 sequence (Figure 3). One truncated mutant (ER/B3Δ1237) deleted the three most C-terminal YXXM motifs containing Tyr-1257, Tyr-1273 and Tyr-1286. ER/B3Δ1237 mediated a weaker association with p85 than was observed with ER/B3. A more pronounced effect was observed with a truncation (ER/B3Δ1204) that removed an additional YXXM sequence corresponding to the loss of Tyr-1219. A third truncation, which resulted in the loss of Tyr-1194 and left only one YXXM motif (Tyr-1051) in the C-terminal domain of ErbB3, produced ER/B3Δ1121; this associated very weakly with p85. Because the accumulated loss of YXXM motifs correlated with progressively weakened associations between ER/B3 and p85, these results suggested that multiple YXXM motifs in ER/B3 contributed to the association with p85.

To study the roles of YXXM motifs further, we generated a mutant ErbB3 protein (ER/B3-6F) that contained Tyr → Phe substitutions at all six YXXM consensus sites. ER/B3-6F was unable to interact with p85, as expected (Figure 3). Consequently we constructed several YXXM add-back mutants. Here individual Tyr residues previously mutated to Phe in ER/B3-6F were restored to Tyr. Interestingly, the ER/B3-1051Y and ER/B3-1194Y add-back mutants, which contained single YXXM consensus sites for p85 binding, were able to interact with p85, albeit extremely weakly when compared with ER/B3. Furthermore the interaction with p85 was considerably augmented when two YXXM motifs were present in ER/B3, as was observed with the double add-back mutant ER/B3-1051/1194Y. These sites were not unique in interacting with p85 because a double mutant, ER/B3-1051/1194F, which contained Phe residues substituted for Tyr at positions 1051 and 1194, but retained the four C-terminal YXXM consensus sites, was able to mediate a stronger interaction than did ER/B3-1051/1194Y. In fact, as

suggested by analysis of the deletion mutants, it seemed that the larger the number of YXXM sites present in ER/B3, the stronger the interaction was with p85, regardless of the location of the sites. It also seemed from an examination of ER/B3-6F that at least one YXXM motif was required for binding and that other Tyr residues in ErbB3, which occur in consensus binding sites for other SH2 domain-containing proteins [29], could not substitute. Additionally, it seemed that multiple YXXM motifs co-operated in p85 binding, because add-back mutants containing multiple motifs associated in a more than additive manner compared with single-site add-back mutants. The mutant proteins showed expression levels similiar to that of ER/B3 (Figures 4A and 4B).

Association *in vitro* of the p85 SH3 domain with ErbB3

To investigate further the role of the p85 SH3 domain in interactions with ErbB3, we generated GST–p85 fusion proteins containing either the p85 SH3 domain (GST–SH3), an SH3 domain carrying the Asp-21 → Asn substitution (GST–SH3*), an SH3 domain with a small proline-rich sequence of the Bcr domain appended (GST–SH3-pro), or the contiguous SH3 and Bcr domains (GST–SH3-bcr) (see Figure 5). GST or GST–p85 fusion proteins were incubated with a recombinant ErbB3 cytosolic domain protein, after which the GST fusion proteins were precipitated with glutathione–agarose. ErbB3 that associated with the GST fusion proteins was detected by SDS/PAGE and Western immunoblotting. Indeed, an interaction was detected between GST–SH3 and ErbB3 (Figure 5), although this interaction was much weaker than that observed with the full-length GST–p85 protein and phosphorylated ErbB3 (results not shown). In contrast, the interaction of the mutant GST–SH3* with ErbB3 was almost undetectable. Interaction of GST–SH3-pro and GST–SH3-bcr with ErbB3 was also much weaker than that of GST–SH3, which suggested that the proline-rich sequences contained in p85 might compete in SH3 domain binding with the proline-rich sequence found in ErbB3.

DISCUSSION

The results of this study show that the association of ErbB3 with the p85 subunit of PI 3-kinase is mediated predominantly by interactions beteween the SH2 domains of p85 and YXXM motifs within the C-terminus of ErbB3, as previously suggested [22]. Other potential Tyr-phosphorylation motifs in the ErbB3 C-terminus do not seem to substitute for the conserved YXXM consensus p85 binding sites, in contrast with one study in which low-stringency protein–protein interactions were observed between EGFR phosphotyrosine sites and SH2 domain-containing proteins [34]. Our results also suggest that multiple Tyr residues within YXXM motifs can interact with the tandem SH2 domains of p85. Spatial relations between these Tyr residues could in principle determine optimal p85 binding [35]. Tyr-1257, Tyr-1273 and Tyr-1286 are separated by fewer than 20 residues each and could be ideally spaced for associating in concert with tandem SH2 domains [35]. Tyr-1051 and Tyr-1194, however, are separated by 142 residues and, from our results, still seemed to co-operate in binding p85. This suggests flexibility in the binding of p85 tandem SH2 domains to differentially spaced Tyr residues as hypothesized [35], in contrast with the relatively rigid spatial requirement of the tandem SH2 domains of SHP-2 [36] and ZAP-70 [37]. A tandem interaction of the SH2 domain with ErbB3 might optimize PI 3-kinase activation by ErbB3. It has been observed that bisphosphopeptides are more potent in stimulating PI 3-kinase activity when compared with monophosphopeptides [38–40].

Differential phosphorylation of Tyr residues in YXXM consensus sites within the ErbB3 C-terminus could ultimately determine which of these sites interact with p85. Little is currently known about the stoichiometry of phosphorylation of these various Tyr residues. Co-operation of the tandem SH2 domains of p85 in high-affinity binding to ErbB3, as we have apparently observed here (see Figures 2 and 3), would require multisite phosphorylation of ErbB3 molecules. However, we have not independently determined which ErbB3 Tyr residues were phosphorylated in the context of the yeast two-hybrid system, or whether individual ErbB3 molecules were phosphorylated on multiple Tyr residues. It is therefore possible that particular YXXM sites were predominantly phosphorylated and responsible for strong ErbB3 associations with p85, or alternatively that Tyr phosphorylation was distributed relatively equally between the YXXM sites. Our preliminary studies of COS7 cells transfected with ErbB3 mutants containing single YXXM sites have shown that p85 is capable of associating with any of the YXXM sites occurring in the ErbB3 C-terminus, which indicates that each of the YXXM sites in the ErbB3 C-terminus is potentially phosphorylated *in vivo* (N. J. Hellyer and J. G. Koland, unpublished work).

The presence of a consensus SH3 domain-binding motif in ErbB3 led us to investigate the roles of this motif and the p85 SH3 domain in ErbB3/p85 interactions. Deletion of the p85 SH3 domain or substitution of an SH3 domain residue critical for peptide recognition significantly decreased the interaction of ER/B3 and p85 (Figure 2). However, a similar effect was observed when examining the interaction of p85 with the insulin receptor cytosolic domain in the yeast system (results not shown), although the insulin receptor possesses no obvious candidate SH3 domain-binding sequences within its cytoplasmic domain. In addition, deletion of the PPRPPRP candidate SH3 domain-binding motif of the ErbB3 C-terminus did not significantly alter the ER/B3 interaction with p85 (Figure 3). Together these results imply that the potential interaction between this ErbB3 proline-rich sequence and the p85 SH3 domain does not contribute significantly to the overall affinity of the ErbB3/p85 interaction.

The decrease in the apparent affinity of the ErbB3/p85 interaction observed on deletion or mutation of the p85 SH3 domain might have been a consequence of the self-association of the p85 SH3 domain and a proline-rich region within the Bcr region of p85 [15], an interaction that could modulate the affinity of p85 SH2 domains for phosphotyrosine-containing substrates. It would also be possible that ligation of p85 SH2 domains by phosphotyrosine-containing peptides could exert a reciprocal effect on the SH3 domain, possibly reversing its intramolecular association with the Bcr domain and enhancing its accessibility to other signalling molecules. In the Ras-Gap/Rho-Gap interaction, it seems that when the tandem SH2 domains of Ras-Gap bind to phosphorylated Tyr residues of Rho-Gap, the conformation of Ras-Gap changes such that there is a 100-fold increase in the accessibility of the target-binding surface of its SH3 domain [41].

Our experiments *in vitro* provided evidence for intramolecular p85 interactions, and furthermore suggested that interactions between the p85 SH3 domain and proline-rich Bcr domain sequences could negate any positive binding contribution attributed to interactions between the p85 SH3 domain and the proline-rich sequence of the ErbB3 (see Figure 5). Hence these results leave open the possibility that ErbB3/p85 SH3 domain interactions do occur and that these interactions are of regulatory significance. For example, the disruption of an intramolecular p85 Bcr/SH3 domain interaction by the ErbB3 proline-rich sequence could modulate the function of p85, perhaps by

regulating its binding to or activation of low-molecular-mass GTP binding proteins such as Cdc42Hs [15,42] or by regulation of the associated p110 catalytic domain.

Other investigators have shown that yeast two-hybrid fusion proteins can be phosphorylated within the yeast two-hybrid system when co-expressed with either the platelet-derived growth factor receptor or Src protein tyrosine kinase domains [43,44]. In those studies, protein tyrosine kinase domains were co-expressed to phosphorylate mammalian signalling proteins that otherwise would not be significantly phosphorylated in yeast cells. This technique allowed investigators to screen for target proteins that interact with phosphorylated Tyr motifs occurring in these signalling proteins. Similarly, our strategy, in which an active protein tyrosine kinase domain was substituted for the otherwise inactive kinase domain of ErbB3, provided us with a functional chimaeric EGFR/ErbB3 protein that was used in mapping the sites of interaction between ErbB3 and the p85 subunit of PI 3-kinase. This strategy could be generally applicable to the investigation of other protein–protein interactions mediated by phosphorylation of Tyr residues. The ER/B3 construct could also be used to screen for novel proteins that interact with ErbB3 by use of the yeast two-hybrid approach.

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